

**A STUDY OF THE MECHANISM BEHIND THE HLA-DO  
ACCESORY MOLECULE AND ITS GREATER ROLE IN  
CLASS II ANTIGEN PRESENTATION**

by

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## **Abstract**

Antigen presentation is the first step in generating an adaptive immune response to a foreign pathogen. The antigen processing system digests antigens into small peptide fragments, which are bound to major histocompatibility complex (MHC) molecules to be presented to T cells. Our study focuses on the MHC class II (MHC II) system, which is responsible for presenting antigens to CD4<sup>+</sup> T cells. Here, we examine the function of the MHC II accessory molecule HLA-DO (DO), and its interaction with another accessory molecule HLA-DM (DM), to regulate the nature of the peptides presented to CD4<sup>+</sup> T cells. Although the genes encoding the DO proteins are evolutionary conserved in the MHC of mammals, their functions have not been well studied. To directly examine DO function, we designed a recombinant soluble DO heterodimer and tested its effect on peptide binding in vitro. The leading model proposes that DO inhibits the effects of DM, but we show that DO can interact directly with the MHC II molecule HLA-DR1 (DR1) and have both inhibitory and enhancing effects depending on the affinity of DR1 for the peptide. Based on our observations, DO only regulates peptide association but not dissociation. We constructed a molecular model for the mechanism of DO in which DO binds to the peptide-receptive MHC II molecules that DM generates, either by dissociating the bound peptides, or opening the empty groove. This model was verified through Surface Plasmon Resonance (SPR) experiments in addition to specific peptide-

binding studies. We conclude that the role for DO is not to inhibit the function of DM, but rather to enforce it, adding another layer of control on peptide selection.

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# **Chapter One**

## **Introduction**

*\* Parts of this chapter are adapted from a published review co-authored by Yuri Poluektov, titled “HLA-DO and its role in antigen presentation” [Frontiers in Immunology, August 2013]*

T cells are of critical importance in the initiation of an adaptive immune response. They are the first cells to detect the presence of foreign antigens and signal an immune cascade to begin fighting an infection that cannot be subdued by the innate system. There are at least two classes of T cells: CD8<sup>+</sup> T cells are primarily responsible for destroying cells containing internal pathogens, and CD4<sup>+</sup> T cells that deal with external pathogens in addition to enhancing the immune response and helping B cell to initiate the production of antibodies against the pathogen. T cells are activated upon recognition of a peptide of a given antigen bound to the groove of Major Histocompatibility Complex (MHC) proteins presenting fragments of antigenic proteins. When a pathogen is introduced into the body of the host, be it a virus, bacteria or even a multicellular parasite, some of its antigenic proteins are processed by the antigen processing system. The processing system digests the full protein leaving behind only small peptide fragments. Those fragments are then bound to MHC molecules and presented to T cells. If the antigenic protein is sufficiently different from peptide fragments generated from

self-proteins T cells will respond and initiate an immune reaction. Similar to most biological systems, MHC molecules do not act alone and are constantly supported by various accessory and processing molecules comprising the antigen presentation system. Even a small dysfunction in one of the accessory molecules could lead to an improper stimulation of T cells resulting in the onset of disease. By studying the molecular mechanism of the accessory molecules, as well as their modes of interaction among them, we hope to be able to manipulate the presentation of antigens to T cells; i.e., suppress the presentation of self-reactive peptides that may result in autoimmune pathologies, or enhance the presentation of foreign antigens to fight pathogens.

### **Class II antigen processing**

Antigens are either handled by the MHC class I or class II antigen processing systems. Class I molecules are expressed virtually by all nucleated cells while class II is only utilized by professional Antigen Presenting Cells (APCs) – Macrophages, B cells and Dendritic cells, as well as thymic epithelial cells. MHC class I molecules are primarily responsible for presenting peptide fragments of proteins derived from endogenous sources while MHC Class II process exogenous proteins from the extracellular space. Cross-presentation can occur between the two systems and peptides from extracellular antigens can be presented by class I molecules and vice versa. However, it is only the class I molecules

that present antigenic peptide fragments to “cytotoxic” CD8+ T cells, while MHC II molecules present their peptides to “helper” CD4+ T cells. Both classes are equally important for the immune system, but our research project focuses on the Class II system and its accessory molecules in particular.

MHC Class II molecules (MHC II) are transmembrane glycoproteins consisting of one  $\alpha$ -chain of approximately 34 kD and one  $\beta$ -chain of approximately 28 kD combined into a heterodimer<sup>1</sup>. Classical MHC II molecules form a receptive groove in between their alpha and beta chains that is able to bind peptides and present them on the cell surface. Non-classical MHC II are non-polymorphic and serve as chaperones and accessory proteins helping in folding, transport, antigenic peptide loading, and editing. The most well studied MHC II accessory molecules, while also composed of transmembrane heterodimeric glycoproteins, do not form a receptive groove that can bind peptides for presentation to T cells and are not transported to the cell surface. Instead accessory molecules interact with classical MHC II to affect its binding of peptides. As is the case with the accessory molecule HLA-DM(DM) that greatly facilitates the loading of antigenic peptides into the MHC peptide-binding groove. We and others have shown that DM interacts with MHC class II and induces conformational changes in the MHC molecule that cause the release of the bound peptide and the generation of a peptide-receptive

conformation<sup>2,3</sup>. The effect of DM on the immune system can be visualized in knockout mice through their display of altered antigen presentation, which in some cases is associated with susceptibility to autoimmunity<sup>4-8</sup>. HLA-DO (DO) is another class II accessory molecule, which unlike DM does not have a visible effect on antigen presentation. One of its unique qualities is that DO stably interacts with DM and can even be co-precipitated as a complex. In fact, the transport of DO out of the ER depends upon its assembly with DM<sup>9</sup>, indicating its importance in class II antigen presentation. The study of DO presents a unique opportunity to discover a new non-invasive method to alter MHC class II antigen presentation. A mechanism to regulate the function of DO if developed, could be used in therapy without the fear of causing systemic anomalies that occur in DM knockout models. At the same time, the function of DO could be inhibited or enhanced to reduce specific pathological conditions.

### **Discovery of DO and its variable tissue expression**

One of the inherent difficulties in studying MHC class II molecules is the fact that alpha and beta chains of the molecules do not, in many cases, reside in close proximity to each other. This is especially true for DO. When first discovered, DO beta chain was thought to be an alternative beta chain for MHC class II molecule I-A and designated as A $\beta$ 2<sup>10</sup>. It was not until years later that the corresponding DO alpha chain was found in a study that used an antibody against the predicted cytoplasmic tail of

the beta chain to pull down the entire molecular complex and separate it by two-dimensional gel electrophoresis<sup>11</sup>. In addition, the same study discovered one of the most unusual features of DO – its variable tissue distribution. Unlike MHC II molecules and DM which were expressed in all professional APCs, DO was expressed only in B cell dependent areas of secondary lymphoid tissues and a few sparse locations in the thymic medulla. In addition, it was later discovered that the expression of DO in B cells was up- or down-regulated depending on the stage of development and activation<sup>12,13</sup>. Only after migration to the spleen do B cells express detectable levels of DO, which are maintained in all transitional B cell subsets and mature cells. However, this expression level is significantly down-regulated after B Cell Receptor (BCR) stimulation and migration into germinal centers.

Studies of human DO analog produced similar results with expression in B cells and thymic epithelium<sup>9,14,15</sup>. Specifically, DO is expressed in epithelial cells ringing the Hassall's corpuscles (HC) structures, which do not exist in mice as well defined structures. Little is known about the true purpose of HCs in the human immune system, although some studies have linked them to the generation of regulatory T cells and autoimmune disease<sup>16</sup>, resulting in speculation about the role of DO in autoimmunity. The HC bodies are the only sites within human thymic medulla where dying thymocytes could be detected outside of the thymic

cortex, which implies an important mechanism in negative selection. More recently, other differences in mouse and human levels of DO expression have been found in certain subsets of dendritic cells (DCs). Most notable are the BDCA-3<sup>+</sup> subset of human blood plasmacytoid DCs, which uniformly express DO, subpopulations of BDCA1<sup>+</sup> CD11c<sup>+</sup> DCs, and tonsillar interdigitating DCs<sup>17</sup>.

The variable expression of DO compared to MHC II molecules and DM, as well as its up- and down-regulation during the B cell cycle, hint at DO's ability to perform a unique function in the MHC class II processing system. DO is evolutionary conserved among mammalian species, in one case it was even observed that it has been under a strong selection for its conservation<sup>18-20</sup>. It has even been reported that a point mutation in the human HLA-DO $\alpha$  gene was linked to a susceptibility to rheumatoid arthritis (RA)<sup>21</sup>, suggesting that the effects of DO on the immune system are far reaching.

### **Functions of DO observed in vivo**

Despite its evolutionary importance, DO has not yet been directly implicated as a significant factor in any disease. At first it appeared that DO had a distinct negative effect on antigen presentation. Cell lines used as surrogate APCs through transfection of DR1, DM and DO genes showed a higher abundance of CLIP bound to DR1 than in the absence of

DO<sup>22</sup>. These results suggested that DO was an inhibitor of DM, specifically regulating DM's ability to dissociate CLIP and promote the binding of other antigenic peptides. This theory does not, however, take into account the fact that B cells from DO knockout mice expressed CLIP at similar levels as their wild-type counterparts<sup>23,24</sup>. In general, results from transfected cells were contradictory to those found in vivo and no experiment could determine the exact effects that DO had on antigen presentation<sup>23-25</sup>. One of the biggest breakthroughs in studying the effects of DO in vivo occurred when investigators began to overexpress DO in CD11c<sup>+</sup> DCs. Those mice were crossed with non-obese diabetic (NOD) mice to generate NOD mice with overexpressed DO in DCs (NOD.DO)<sup>26</sup>. The results were quite overwhelming in that NOD.DO mice failed to develop diabetes even after 50 weeks of observation. They did, however, develop diabetes upon receiving donor T cells from wild type NOD mice. When NOD.DO T cells were transferred into NOD.*scid* hosts lacking T and B cells, mice developed diabetes. Yi *et. al.* went through great lengths to document that the overexpression of DO did not suppress the generation of diabetogenic T cells or increased the activation of T regulatory cells (Tregs). Instead, DO altered the frequency at which diabetogenic T cells were activated through modulating antigen presentation. Another study found that DO knock-out mice could spontaneously develop high titers of antinuclear antibodies (ANAs), indicative of a mouse model of autoimmune systemic lupus

erythematosus<sup>27</sup>. The mice did not, however, develop an autoimmune pathology associated with lupus, but showed a reduced capacity to present exogenous antigens to helper T cells.

The results observed in transgenic NOD mice and DO knockouts, while significant, did not provide insights into the mechanism of DO. This allowed the original paradigm of DO as an inhibitor of DM to largely remain unopposed. The only experimental evidence to the contrary came from a study which looked at the ability of B cells in DO knockout and wild-type mice to enter germinal centers (GCs)<sup>28</sup>. The study reasoned that because the expression of DO was down-regulated upon entry to GC, DO may be affecting the ability of B cells to enter the GCs. Authors proposed that if DO had a measurable effect on the presentation of antigens on the cell surface, it would also have had some effect on the ability of B cells to receive CD4<sup>+</sup> T cell help, and hence enter the GCs. To test this hypothesis a 1:1 mixture of DO knockout and wild-type B cells specific for the 4-hydroxy-3-nitrophenyl acetyl ligand (NP) were adoptively transferred to B6 recipient mice. Mice were then immunized with NP-linked chicken gamma globulin (CGG), and then the abundance of DO knock-out and wild type B cells in the GCs were measured twenty days later. The study found that DO knockout cells outnumbered wild type B cells by a ratio of 3 to 1. However, when NP-linked ovalbumin (OVA) was used as an antigen, the effect was reversed. This time wild



type B cells expressing DO outnumbered the DO knockout cells in the GCs. These observations demonstrate that the effect of DO on antigen presentation can vary depending on the antigen, and the theory that DO is an inhibitor of DM does not explain how DO works.. The function of DO is extremely hard to pinpoint with in vivo models due to the complexity of the antigen presenting system, hence a need for biochemical and structural studies of simplified in vitro MHC II systems is apparent.

### **Mechanistic and structural studies of DO**

Most mechanistic studies to date start with the assumption that DO inhibits DM function, and as such are limited in their interpretation. While DM inhibition may be one of DO's functions, as it was observed in some of the earliest biochemical assays performed with purified DM/DO complexes<sup>22,29</sup>, it should not be viewed as the only role of DO in antigen processing. One study suggested that the ability of DO to inhibit DM was pH dependent – evident only in the pH 5.5 – 6.0 range and absent in the pH 4.5 – 5.0 range<sup>23</sup> – which might be a clue toward DO's ultimate role. Many studies, however, continue to view DO as an inhibitor of DM<sup>30,31</sup>.

An examples of DO as an inhibitor of DM was generated with the 3-D crystal structure of the DM/DO complex<sup>32</sup>. Through biochemical experiments Guce *et. al.* showed that binding of DO to DM reduced its

ability to enhance binding of a variant of HA-306-318 peptide of the influenza Texas77 strain to the MHC class II molecule HLA-DR1 (DR1), or to dissociate CLIP. More importantly, the interface of DM that was primarily utilized in its interaction with DO was the same as the one used by DM to exert its effect on DR1, as is evident from the DM/DR1 crystal structure<sup>33,34</sup>. Thus the structure of the DO in complex with DM superimposed well with the known structure of DR1<sup>35</sup>. The same held true for the conformation of DM when compared with the previously solved unbound DM structures<sup>36,37</sup>. These structures suggest that DO acts as a structural mimic of MHC II, which upon binding to DM prevents it from functioning as an accessory molecule for MHC II. While the interface between DM and DO may be the same or similar as that between DM and DR1, it is important to emphasize that the stability of each complex is different. While the DM/DO complex is highly stable under most conditions, the generation of the DM-DR1 complex requires conformational changes to occur in the DR1 molecule, including the generation of a peptide-receptive-DR1 complex<sup>38,39</sup>. Even though the evidence of the ability of DO to inhibit the function of DM is strong, it would be premature to assume that this is the only function of DO. It is unlikely that this accessory molecule survived this long in multiple species without accumulating any polymorphism if its only function is to disable another accessory molecule.

Alternative models to explain the function of DO have been proposed in the past. Unfortunately, not much work has been done to test these models. One notable study reported that DM/DO complexes purified from the human spleen had a positive effect on the loading of HA peptide onto DR4, DR1 and DR3 molecules as compared to purified DM alone<sup>40</sup>. Interestingly, the study replicated this same result with a recombinant form of DO instead of the cell purified molecules. More importantly, the study included elution experiments from DR4 molecules using a predetermined array of eight peptides. The addition of DO changed the repertoire of the eluted peptides as compared to DM. In the presence of DM, four of eight peptides were predominantly loaded onto the DR4 molecules, but when both DM and DO were present, a different set of four out of eight peptides was detected. These findings strongly suggest that like DM, DO can up- or down-modulate the presentation of certain peptides. With that mindset our lab decided to undertake a study of DO in a cell-free system to determine the exact conditions necessary for DO to inhibit the association as well as dissociation of peptides to DR1<sup>41</sup>.

### **DO can both inhibit and enhance peptide loading on DR1**

Our kinetic binding assays showed that in addition to inhibiting the binding of some peptides to DR1, DO can also enhance the binding of others. In Chapter 2, working with soluble recombinant DM, DO and DR1 constructs we show that DO enhanced the binding of the HA-306-

318 as well as H5N1-HA1(259–274) peptides to DR1, while diminishing the binding of CII(259–273) and HA-306-318 peptide derivatives with weak anchoring residues positioned in the P1 pocket of the MHC II molecule. This characteristic of DO is detected both in the presence and the absence of DM, and even more surprising, in conjunction with DM's ability to enhance peptide presentation. Interestingly, the peptides selected for by DO seem to take priority over DM's choices, when it comes to the inhibitory effects of DO. The regulatory activity of DO manifests itself during the association of peptides to DR1 and is undetectable during the dissociation phase. The effect of DO is detectable in both its free form, as well as in the DM/DO complex generated by producing both proteins in the same cells. This indicates that although DO will probably inhibit the effect of DM in the DM/DO complex, this binding event does not diminish the activity of DO. We suggest a model in which DM is used to generate a peptide-receptive conformation<sup>3,42,43</sup> in MHC II molecules, which DO then uses as a substrate to perform an additional round of selection by limiting which peptides can bind to the peptide-receptive MHC II.

### **DO interacts directly with DR1 and not through modulating DM**

In Chapter 3 we confirm our model by testing the ability of DO to interact with peptide-receptive DR1 molecules. We present evidence that DO can interact with DR1 in the absence of DM by showing both the enhancing

and diminishing effect of DO on peptide-receptive DR1 molecules. In addition, DO did not lose its ability to diminish the binding of peptides to a mutant DR1 molecule stabilized in a peptide-receptive conformation. As a validation of this interaction we detected the binding of DO molecules to peptide-receptive DR1 through SRP experiments on the BIAcore 1000 instrument. DO bound to both peptide-receptive mutant DR1 molecules as well as wild-type DR1 in the presence of DM, but failed to bind to HA-306-318 peptide loaded DR1. This allowed us to propose a hypothetical model for the mechanism of DO interaction with MHC II, and we suggest that DO only interacts with MHC II in a peptide-receptive conformation, normally generated through the action of DM. Once the MHC II is receptive and free of peptides, a DO/MHC II complex is formed allowing for more efficient binding of peptides with strong anchoring residues. Peptides with weak anchoring residues, especially in the P1 pocket, do not bind to the DO-DR1 complex as efficiently. Since DM equally enhances the binding of all peptides to DR1, the ability of DO to select for some peptides over others may in fact seem like an inhibition of DM's function, while the actual role of DO is greater. We propose that DO is not an inhibitor of DM but a "second editor of the peptide repertoire" presented by MHC II. As such, DM serves as the primary editor of the peptide repertoire by dissociating CLIP and other weak-anchored, or "DM-sensitive", peptides from MHC II and leaving it in a receptive conformation. DO then acts on the resulting receptive DR1 to add its

own layer of selection by allowing only peptides with strong anchoring residues, mostly “DM-insensitive,” to bind. Thereby DM and DO work together to select for the “best” peptides to be presented by MHC II molecules to T cells for stimulation.

### **DO changes the selection of antigens presented by DR1**

To validate our mechanistic findings we examined the ability of DO to regulate the processing of a full length antigenic protein into peptide fragments selected by DR1 for presentation. In Chapter 4 we utilize our minimalistic cell-free system<sup>44</sup> to determine sequences of peptides that remain bound to the DR1 molecule upon full antigenic processing in the presence and absence of DO. We found that the presence of DO had little effect on selecting a known immunodominant antigenic peptide from the H5N1-HA1 protein, which was mainly selected by the actions of DM. In contrast, we detected a significant decrease in the amount of a known DR1 antigenic peptide epitope selected by DR1 molecules in the presence of DO or even both DM and DO when compared to DM alone. As a validation of our biochemical experiments where DR1 molecules were exposed to only a handful of synthetic peptides to determine the effects of DO, we find that DO has a translatable effect on the selection of immunodominant peptides from a full length antigenic protein.

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## Chapter Two

### **DO can have variable effects on peptide binding to DR1, depending on the peptide structure**

*\* Parts of this chapter are adapted from a published article co-authored by Yuri Poluektov, titled “HLA-DO as the optimizer of epitope selection for MHC class II antigen presentation” [PLoS One, August, 2013]*

#### **Introduction**

DO is an accessory molecule of the MHC Class II system that has had an evolutionary presence in the MHC locus in some of the earliest mammalian species<sup>1-3</sup>. But unlike DM the function of DO has remained elusive. Upon synthesis in the endoplasmic reticulum (ER), membrane bound classical MHC II molecules assemble into heterodimers composed of an alpha and beta chain. To prevent the MHC II molecules from binding to proteins and peptides present in the ER, their peptide binding groove is blocked by a tight interaction with the membrane bound protein known as the Invariant chain (Ii), which also increases the stability of the MHC II molecules. When transported to the specialized endosomal compartment, known as MIIC, where class II molecules are exposed to antigenic proteins, Ii is digested away and only a small fragment of it called CLIP remains bound to the MHC II groove<sup>4</sup>. One of the main functions of DM is to dissociate the CLIP peptide from MHC II so that antigenic peptides may bind to their grooves<sup>5-8</sup>. This process is of

utmost importance to class II antigen presentation and its dysfunction is visualized in DM knockout experiments<sup>9-12</sup>. DO knockout experiments, on the other hand, never resulted in a clear phenotype. This is surprising, given that DO was originally found to form a strong complex with DM<sup>13</sup>. It was even proposed that the function of DO is to inhibit DM, but that theory could never be completely confirmed or disproved<sup>14</sup>.

In order to address the confusion in the field our lab has performed a series of biochemical experiments to discern the function of DO. Unlike most studies dealing with cells<sup>15</sup> and in vivo DO knock-out models<sup>16-18</sup> we took a minimalistic approach to determine the effects of DO on peptide loading. The MHC II system is extremely complex to study in vivo and there are too many triggers that can be engaged by removing or altering an accessory molecule in the system. This creates a complicated chain of events that obscures the antigen presentation mechanism, making it impossible to distinguish between cause and effect. With this in mind, we decided to examine the effects of DO in vitro, as we have in the past for other MHC Class II molecules<sup>19-22</sup>, by examining the effects of recombinant soluble DO molecules (missing the transmembrane domain) on the binding of peptides to recombinant DR1 in the presence or absence of DM. We utilized this technique to determine how DO regulates the loading and dissociation of peptides from DR1. Our results

suggest that the effect of DO is variable and most likely dependent on the ability of DM to dissociate the peptide.

## **Materials and methods**

### **A. Production of soluble recombinant MHC class II proteins**

Soluble DR1, DM and DO proteins were expressed and purified as originally described<sup>23,24</sup>. Baculovirus DNA (BaculoGold; BD Biosciences, San Jose, CA) and transfer vectors carrying the recombinant soluble forms of the alpha and beta chains of DR1, DM and DO were transfected into SF9 insect cells for recombinant virus production, as suggested by the BaculoGold protocol. Hi5 cells, upon reaching a concentration of  $\approx 1 \times 10^6$  cells/ml were infected with the recombinant virus and grown in suspension in shaker flasks for 3 days following infection for protein production. Soluble DR1 was purified from both the cells and culture supernatant using anti-DR1 mAb (L243) coupled resin. For the purification of HLA-DM and HLA-DO, the protocol was modified to use the M2 FLAG (Sigma-Aldrich, St. Louis, MO, USA) and Ni-NTA (QIAGEN, Valencia, CA, USA) affinity columns respectively using gravity flow. Upon elution from the columns DM and DO molecules were concentrated (20-40  $\mu$ M) in Citrate Phosphate pH 6.0 buffer with 0.05% NaN<sub>3</sub> and stored frozen at -80°C in small aliquots. The soluble DR1 heterodimers were kept in PBS pH 7.4 with 0.05% NaN<sub>3</sub>.

## **B. Peptide synthesis and labeling**

Peptides used in our binding experiments were synthesized to a minimum of 90% purity (Global Peptide Services, currently Pi Proteomics, Huntsville, AL, USA), as confirmed by HPLC and mass spec documentation from the company. All peptides in our study were derived from characterized immunodominant HLA-DR1 (human MHC class II) binding peptides. The peptides were labeled with Fluorescein on their N-terminus with either fluorescein-5-maleimide (Molecular Probes), when the peptide had an added Cysteine residue on the N-terminus, or with fluorescein 5,6 succinimidyl ester (Molecular Probes) directly to the N-terminus, as per manufacturers suggested protocol. The labeled peptides were concentrated to 0.05 ml by SpeedVac (Savant Instruments, Inc.) and excess free fluorescein was removed by passing the sample through a Sephadex G-10 column (Sigma-Aldrich). The concentration of the peptide was determined according to the extinction coefficient of Fluorescein ( $83 \text{ mM}^{-1} \text{ cm}^{-1}$ ).

Sequence of peptides used in the study:

**HA(306-318):** CPKYVKQNTLKLAT

(derived from Texas 77 flu strain HA protein peptide)

**HA(Y308A):** PKAVKQNTLKLAT

(Texas 77 flu strain HA protein peptide variant)



**HA(anchorless):** CPKAVKANGAKAAT

(Texas 77 flu strain HA protein peptide variant)

**CII(259-273):** CAGFKGEQGPKEP

(derived from Type II Collagen peptide used to induce arthritis in mice models)

**H5N1-HA1(259-274):** SNGNFIAPEYAYKIVK

(derived from A/Vietnam/1203/2004 flu strain peptide<sup>25</sup>)

### **C. Peptide association and dissociation kinetics**

Peptide binding experiments were performed as described previously<sup>20-22,26</sup>. In brief, we performed all binding experiments at 37°C in Citrate-Phosphate buffer, pH 5.0. Wt DR1 (1  $\mu$ M) was incubated with fluorescent peptides (30  $\mu$ M) in association experiments. For dissociation experiments we used DR1 /fluorescent peptide complexes formed over 3 days incubation in 37°C, a competing non-fluoresceinated HA(306-318) peptide was added to the dissociation reaction at a concentration of 50  $\mu$ M. In kinetics experiments where DO and DM were included, concentrations of 1  $\mu$ M for DM and 4  $\mu$ M DO or DM/DO were used. For each of the experimental combinations of DR1 with DM and DO, the binding of peptide to DR1 was measured at eight different time points: 10h, 5h, 3h, 2h, 1h, 0.5h, 0.25h, 0h, unless otherwise indicated. Excess unbound peptide was removed using Sephadex G-50 size-exclusion spin columns. As a test for complete peptide removal as well as non-specific

binding, all association assays included binding controls of peptide alone or with accessory molecules incubated for over 10 hours at 37°C. Binding control values were included in the corresponding figure legends. Fluorescence signal of peptide/DR1 complexes was measured by a Horiba FluoroMax<sup>®</sup>-3 instrument (Horiba Ltd., Kyoto, Japan) at 514-520 nm with an excitation wavelength of 492 nm and a 2 nm slit width.

#### **D. Surface Plasmon Resonance measurements of protein binding**

SPR experiments were performed as described previously<sup>26</sup> on a BIAcore 1000 instrument (GE Healthcare). Anti-His tag (Invitrogen), Anti-FLAG tag (Sigma-Aldrich), and Anti-HA tag (Sigma-Aldrich) antibodies were immobilized on CM5 chips (GE Healthcare) through standard amine coupling procedures. Excess activated dextran carboxylate groups were capped with 2-amino-ethyl-sulfate (pH 8.0) to decrease the nonspecific binding of MHC molecules to the chip surface at a lower pH<sup>27</sup>. All protein solutions were diluted in the running buffer composed of Citrate-Phosphate pH 5.5 with 150 mM NaCl, 0.005% Tween-20 and 0.05% NaN<sub>3</sub>. Measurements were taken at 27°C with flow rates ranging between 5-10 µl/min. MHC II proteins were injected at concentrations of 4µM unless indicated otherwise.

#### **Data analysis**

All kinetic experiments performed were analyzed with OriginPro 7.0 software. Association data was fitted by a single exponential association equation (BoxLucas1 fit curve):

$$Y = A_1(1 - e^{-x/t})$$

Dissociation data was fitted by a single exponential dissociation equation (ExpDec1 fit curve):

$$Y = A_1 e^{-x/t}$$

Where x is the number of hours the DR1 molecule was incubated with peptide in the presence of accessory molecules and Y is the resulting fluorescence signal that is read in the Fluorimeter. For association kinetics the units of the Y-values were expressed as  $\times 10^3$  Arbitrary Fluorescence Units (AFU). The dissociation Y-values were normalized to the RFU signal of the 0 hour time point and expressed as % of peptide remaining, taking the starting point to be 100%. We opted out of trying to fit our data with double exponential curves, as it was not sensitive enough to make a significant distinction between single and double exponential fits.

## **Results**

### **A. Soluble recombinant DO molecules expressed are properly folded and resemble the structural features of a native DO heterodimer**

Although previously we were successful with our soluble recombinant MHC II molecules, it is always necessary to confirm that a new recombinant molecule is properly folded and structurally identical to its native counterpart. The native form of DO, like all other MHC II, contains a transmembrane domain which links the heterodimer to the membrane. The MHC II transmembrane domain rarely has been implicated in having an effect on the function of the molecules<sup>28</sup>, and can be removed without any detectable consequences. Unfortunately, DO alpha and beta chains do not dimerize as readily as DM or DR1 and even require the presence of DM for proper dimerization and transportation into specialized endosomal MHC class II compartments (MIICs)<sup>13,29</sup>. To solve this, we removed the transmembrane domain of DO in our recombinant molecule and instead replaced it with a Leucine zipper domain (acidic –  $\alpha$  chain, basic –  $\beta$  chain) (**Fig. 1**). We also added a thrombin cleavage site in the alpha and beta chains as well as a Histidine tag on the  $\alpha$  and an HA tag on the  $\beta$  chain for identification and purification purposes. Spacers were inserted in between the new structures to give the alpha and beta chains enough flexibility to form stable heterodimers. Recombinant soluble DO was expressed in Hi5 insect cells and purified via the Ni-NTA (anti-His tag) column. The molecule purified in this way was not ‘clean’ and its alpha (MW  $\approx$  31 kDa before glycosylation) and beta (MW  $\approx$  32 kDa before glycosylation) chains were barely detectable on a silver stain gel (**Fig. 2A**). Since DO was originally found to have a strong association to the

DM molecule<sup>13</sup> we decided to see if our recombinant molecule could form a stable complex with recombinant DM. To ensure the formation of the complex we decided to co-infect the viral constructs of both recombinant DM and DO molecules into the same Hi5 cells. The resulting protein was purified not through an affinity tag, but by the means of a conformation-specific antibody generated upon immunizing mice with DM/DO complexes naturally expressed in B cells<sup>30</sup>. This antibody (Mags.DO5) was covalently coupled to Sepharose beads and used to purify DM/DO complexes through gravity flow. DM/DO was eluted with CAPS buffer pH 11.5 and buffer exchanged into Citrate-Phosphate buffer pH 6.0. The presence of the alpha and beta chain of both DM and DO in the complex was verified via replicate western blots specific for each of the chains (**Fig.2B**)(DO- $\alpha$ -His-tag; DO- $\beta$ -HA-tag; DM- $\alpha$ -FLAG-tag; DM- $\beta$ -C-Myc-tag). As a verification of the Western BLOTs, the presence of the His- and HA-tags on DO in the DM/DO complex was verified through SPR experiments on the BIAcore 1000. Mags.DO5 purified DM/DO complexes bound both the anti-His and anti-HA antibodies immobilized on CM5 chips through amine coupling (**Fig.3A-B**), while soluble recombinant DM and DR1 did not. Finally, as a validation of the proper tertiary structure of our soluble DO, we purified DM/DO complexes with a Ni-NTA column specific against the DO $\alpha$  chain Histidine tag and tested its binding to the conformation specific Mags.DO5 antibody. DM/DO purified with the His-tag bound to immobilized Mags.DO5 at a level of about 600 relative units

(RU). At the same time, Mags.DO5 failed to bind DM alone of His-purified DM/DO that was pre-incubated with soluble Mags.DO5 prior to injection into the BIAcore instrument (**Fig.4**). This indicates that our insect-expressed recombinant DO folds with sufficient similarity to its B cell-expressed counterpart to reproduce the natural epitope. We have exhausted all possible methods of validating the structure of our recombinant DO short of determining a three dimensional crystal structure of the molecule<sup>31</sup>.

#### **B. Storage of DO in a neutral pH buffer leads to its inactivation**

Our first kinetic experiments with our recombinant DO were not successful; DO seemed to have little to no effect on the binding of peptides to DR1 within a 10 hour period. The effects of DM could be visualized within 15-30 minutes of incubation of DR1 with peptide<sup>20</sup>. The first batches of purified DO molecules were stored in PBS, just like DR1, but this storage buffer modified the properties of our recombinant DO, causing it to aggregate. Preserving DO in a buffer of pH 7.0 showed a lower number of DO dimers as determined by a sizing HPLC column (**Fig.5**). At pH 7.0, the amount of 50-60 KDa DO dimer is much lower than if DO is stored at pH 6.0. This was critical for the effect of DO when looking at its ability to diminish peptide binding to DR1 molecules. We tested the effects of DO, both in the presence and absence of DM, on the binding of a type II collagen derived peptide CII(259-273) to DR1. DO

stored at pH 7.4 had no effect on the association of CII(259-273) to DR1 while DO stored at pH 6.0 clearly diminished the binding of peptide (**Fig.6**).

### **C. DO can have both enhancing and diminishing effects on peptide binding to DR1**

After determining the storage conditions for our DO molecules we tested its effect on the kinetics of association and dissociation of peptides to and from DR1. While DO diminished the binding of CII(259-273) peptide to DR1, it had little to no effect on the dissociation of the same peptide from DR1, both in the presence and absence of DM (**Fig.7**). A similar result was produced when testing our DO with a variant HA peptide molecule lacking all of its anchoring positions – HA(anchorless).

HA(anchorless) is a variant of HA (306-318) of influenza immunodominant epitope that dissociates rapidly from DR1. The dissociation process is even further enhanced by the action of DM<sup>32</sup>. Just like with CII(259-273), DO did not change the dissociation rate of HA(anchorless) (**Fig.8**). But the association of HA(anchorless) to DR1 was almost completely inhibited by DO unlike with CII(259-273) peptide, where DO only diminished the binding but did not prevent it altogether. More importantly, the association of HA(anchorless) was inhibited by DO independent of whether DM was present or not.

Our experiments with CII(259-273) and HA(anchorless) peptides demonstrated that DO can have a negative effect on peptide binding, similar to reports from other labs<sup>15,33</sup>. In this case both peptides had weak anchoring residues and were easily dissociated by DM. What came as a surprise was the observation that DO could also have positive effects on the binding of peptides to DR1. When tested with peptides that were not sensitive to dissociation by DM (DM-insensitive), DO showed an enhancing effect in the binding of both HA(306-318) (**Fig.9**) and H5N1-HA1(259-274) (**Fig.10**) peptides. In each case DO had no effect on the dissociation of the peptide, but the association seemed to be significantly enhanced. In addition, the effects of DO seemed to be cooperative with those of DM. Furthermore, even DO alone increased the ability of DR1 to bind peptides nearly to the same level as DM. Since HA(306-318) has a long halftime of dissociation, we performed an extended DR1 dissociation experiment to determine if the effects of DO become visible at time points beyond the 10 hour mark (**Fig.11**). Even after 100 hours of dissociation the amount of peptide bound to DR1 while being in the presence of both DM and DO was not any different than in the presence of DM alone. The soluble DR1 molecules were degrading faster than the accessory molecules could affect the dissociation of peptides bound to them.

**D. DO forms a complex with DM and upon formation the DM/DO complex has the same effect on peptide binding as DO alone**



The ability of DO to bind DM was one of the first observations made in the study of DO<sup>13</sup>. We have verified that DO can form a complex with DM if both molecules are synthesized in the same cell (**Fig.4**), but can soluble DO also form a complex with DM? To examine this notion, we immobilized DO on the surface of a CM5 chip with covalently coupled anti-His Ab. Once the dissociation of DO from the antibody reached a stable phase that did not alter the signal by more than 10RU/min, we injected soluble DM over the immobilized DO. This resulted in DM binding to DO with a signal difference of 490 RU between the start and the end of the injection of DM (**Fig.12A**). A reverse approach with immobilizing DM to an anti-FLAG tag antibody coupled chip surface and injecting DO led to binding of DO that increased with its increasing concentrations (**Fig.12B**). Measuring the difference in RU between the start and the end of the injection of DO allowed us to plot the amount of DO bound to DM (in RU – Y-axis) versus the concentration of DO injected (in  $\mu\text{M}$  – X-axis) (**Fig.12C**). By fitting the points with a Langmuir 1:1 binding isotherm equation:  $Y = \text{Max} * X / (X + K_d)$ , we are able to determine the  $K_d$  of DO binding to DM. Our numbers approximated the  $K_d$  to be  $\approx 0.5 \mu\text{M}$ .

While, even in its soluble form, DO is able to bind DM, we questioned how this might affect the ability of DO to influence peptide binding to DR1. To this end we examined the effect of DM/DO complexes purified

through the DO $\alpha$  Histidine tag on the binding of HA(Y308A) peptide to DR1 (**Fig.13**). HA(Y308A) has a alanine substitution at its critical P1 pocket anchoring residue making it sensitive to dissociation by DM. To our surprise, DM/DO complexes acted exactly like DO alone on inhibiting the binding of the peptide. Even when the DM/DO was supplemented with additional DM, it did not modify the effect of DO. This indicates that DO, even in complex with DM, can still perform its function.

## **Discussion**

Most studies to date have had trouble determining the effects of DO<sup>16,17,34</sup>. The generally accepted model assumes that DO is an inhibitor of DM<sup>14</sup>. Our studies showed that the function of DO is much more complicated than originally thought. The ability of DO to inhibit the binding of certain peptides is unquestionable, but we show that DO also has opposite effects on binding of other peptides. Our peptide association experiments have demonstrated that CII(259-273), HA(anchorless) and HA(Y308A) peptides are diminished or completely inhibited in their binding to DR1 in the presence of DO. As is evident from our dissociation experiments, HA(anchorless) nearly completely dissociates from DR1 within 2-3 hours of incubation with DM or both DM and DO accessory molecules present (**Fig.8**). CII(259-273), on the other hand, is less susceptible to the effects of DM and it could take up to 10 hours to get it

to dissociate completely in the presence of DM (**Fig.7**). The inhibiting effects of DO on CII(259-273) binding to DR1 are much less pronounced than on HA(anchorless), leading us to hypothesize that the effect of DO is linked to the sensitivity of the peptide to dissociation by DM, since the faster the dissociation rate of the peptide, the more inhibition of its binding is caused by DO. If, however, the peptide is virtually insensitive to dissociation by DM, like HA(306-318) and H5N1-HA1(259-274), then the inhibition of DO is reversed and the binding of the peptide to DR1 is enhanced (**Fig.9-10**). This provides evidence for DO not simply being an inhibitor, but an accessory molecule able to edit the repertoire of peptides bound to DR1, while selecting for some peptides and excluding others. The difference between HA(306-318) and HA(Y308A) peptides is only one residue, that stabilizes the peptide in the P1 pocket of the DR1 molecule. By changing this residue we are not only able to adjust the sensitivity of the peptide to DM dissociation, but also the effect DO would have on its binding to DR1 (**Fig.13**). The ability of DO to shift the peptide binding repertoire by both enhancing and diminishing the binding of peptides has been observed before<sup>35</sup>. Kropshofer et al. also attributed the effect of DO on peptide selection to the activity of DM, but the study did not specify a mechanism for this dependence.

It is important to note, that while the binding of CII(259-273) peptide in the presence of DO is diminished (**Fig.7**), the addition of DM in

combination with DO to the reaction is able to restore some of the binding but not to the same level as that of DM alone. When we examine the association of HA(anchorless) to DR1, this is not the case. The inhibition of DO cannot be reversed by the addition of DM (**Fig.8**). This would indicate that the mechanism of action of DO and DM is separate and that the effect of DO is dominant over that of DM. On the other hand, when we observe the ability of DO to enhance the binding of DM-insensitive peptides, we see an almost cooperative effect between the two accessory molecules (**Fig.9-10**). The enhancing effect of DO and DM together produced more DR1/peptide complexes than either DO or DM alone. Although evidence seems to point strongly toward the mechanisms of DM and DO being distinct, we would need additional experiments presented in Chapter 3, to make a definitive statement.

We have demonstrated that while being able to form complexes with DM, DO did not lose its activity. A DM/DO complex molecule has the same effect on the binding of peptides to DR1 as DO alone (**Fig.13**). Guce et al. has provided strong evidence in the analysis of the crystal structure of the DM/DO complex that the interface of the interaction between DM and DO blocks the residues necessary for DM to interact with DR1<sup>31,36</sup>. This does not, however, suggest that DO in complex with DM would also lose its activity, since we do not know the nature of the residues important for the DO function. In addition, the interaction

between DM and DO, although structurally similar to the interaction between DR1 and DM<sup>37</sup>, cannot be considered the same since the stability of the DM/DO complex greatly exceeds that of DM/DR1. DO, while being a structural mimic of DR1, acts as a unique accessory molecule in the MHC class II system imposing its exclusive layer of peptide repertoire editing in addition to the one imposed by DM.

## **Figures**

### **Figure 1. Soluble recombinant HLA-DO.**

Design and sequence of the recombinant soluble HLA-DO  $\alpha$  and  $\beta$  chain constructs.

DO $\alpha$ -Spacer-Thrombin Cleavage Site-Spacer-Leu Zipper (Acid)-Spacer-6xHis Tag

MALRAGLVLGFTLMTLLSPQEAGATKADHMGSGPAFYQSYGASGQFTHEFD  
EEQLFSVDLKKSEAVWRLPEFGDFARFDPQGGLAGIAAIKAHLDILVERSNRSRAI  
NVPPRVTVLPKSRVELGQPNILICIVDNIFFPVNITWLRNGQTVTEGVAQTSFYSQ  
PDHLFRKFHYLPFVPSAEDVYDCQVEHWGLDAPLLRHWELQVPIPPDAMEGTG  
GGGGGSSSADLVPRGS TTAPSAQLEKELQALEKENAQLEWELQALEKELAAQGS  
GSHHHHHH

DO $\beta$ -Spacer-Thrombin Cleavage Site-Spacer-Leu Zipper (Acid)-Spacer-FLAG Tag

MAISGVPVLGFFIIA VLSAQESWATDSPEDFVIQAKADCYFTNGTEKVQFVVRF  
IFNLEEYVRFDSDVGMFVALTKLGQPDAEQWNSRLDLLERSRQAVDGVCRHNY  
RLGAPFTVGRKVQPEVTVYPERTPLLHQHNLLHCSVTGFYPGDIKIKWFLNGQEE  
RAGVMSTGPIRNGDWTFQTVMILEMTPELGHVYTCLVDHSSLLSPVSVIEWRAQ  
SEYSWRKSRGGSGGGSSSADLVPRGS TTAPSAQLKKKLQALKKKNAQLKWKL  
QALKKKLAQGS GSYPDVDPDYA

**Figure 2. Recombinant soluble DO characterization.**

(A) Gentle SDS (0.2% SDS) gel of purified soluble DO and DR1 on a 12% Tris-HCl gel.

(B) Soluble co-expressed DO/DM complex was purified using Mags.DO5 monoclonal antibody affinity column. Four replicate samples were resolved on Bis-Tris SDS-PAGE gels in decreasing protein concentrations. Gels were blotted to PVDF membranes and stained for DO (left, anti-His and anti-HA) and DM (right, anti-FLAG and anti-c-Myc) specific tags.



**A**

MW (KDa):

97.4 -

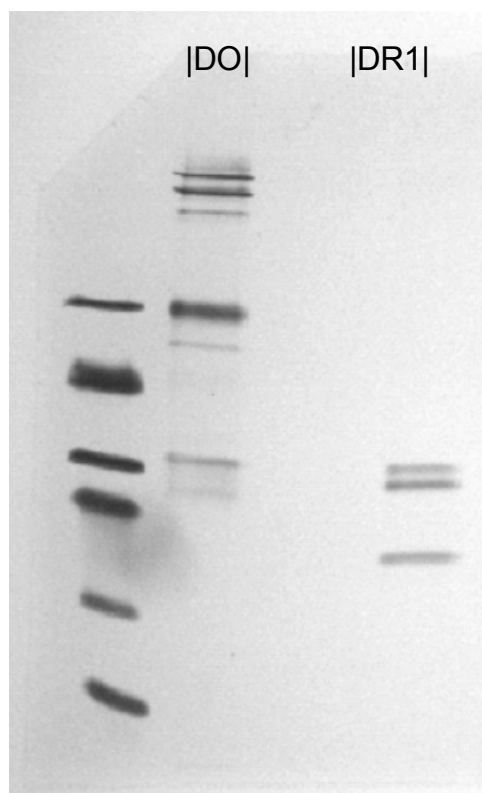
66.2 -

45.0 -

31.0 -

21.5 -

14.4 -

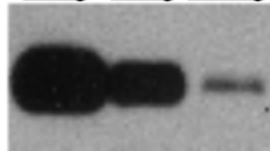
**B****HLA-DO**

DM/DO co-expression

0.2 ug 0.1 ug 0.05 ug

HLA-DO  $\alpha$ -chain

0.2 ug 0.1 ug 0.05 ug

HLA-DO  $\beta$ -chain**HLA-DM**

DM/DO co-expression

0.2 ug 0.1 ug 0.05 ug

HLA-DM  $\alpha$ -chain

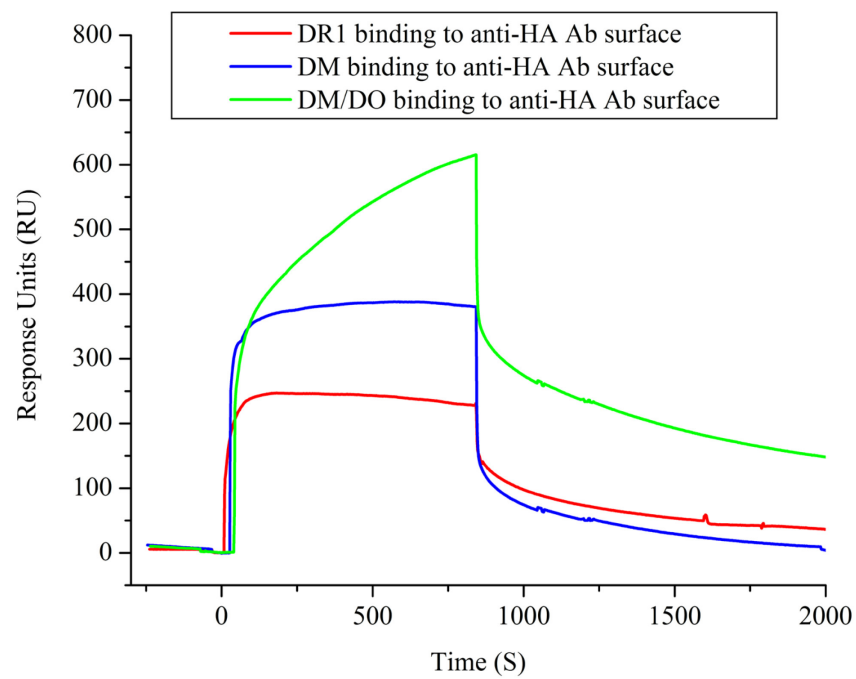
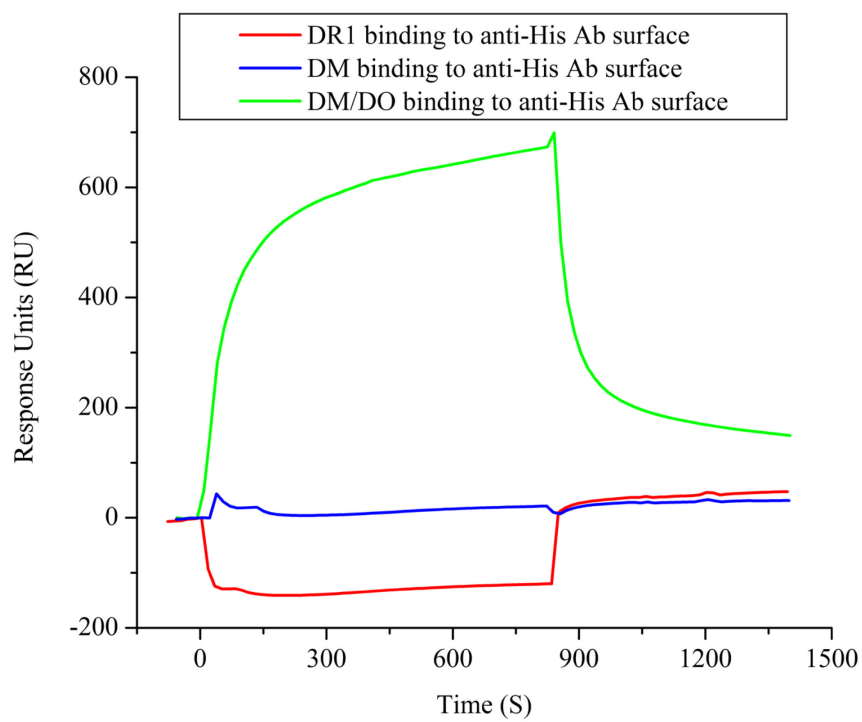
0.2 ug 0.1 ug 0.05 ug

HLA-DM  $\beta$ -chain

**Figure 3. Recombinant DO Characterization on BIAcore 1000.**

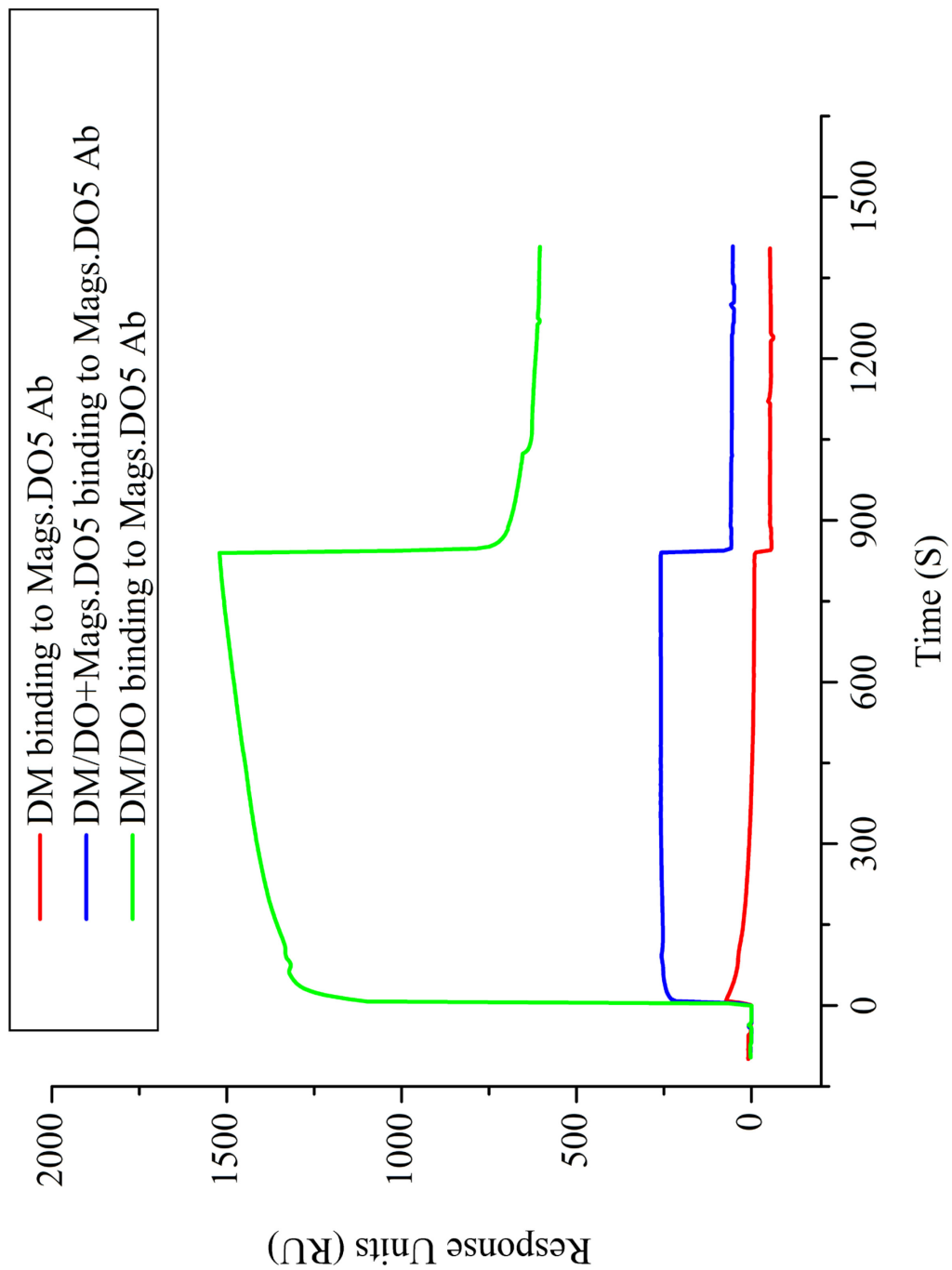
(A) SPR sensograms of DR (red trace), DM (blue trace) and co-expressed DM/DO complexes (Mags.DO5 purified) (green trace) binding to anti-HA antibody coupled chip surfaces.

(B) SPR sensograms of DR (red trace), DM (blue trace) and DM/DO (Mags.DO5 purified) (green trace) binding to anti-His antibody coupled chip surfaces.

**A****B**

**Figure 4. Recombinant DM/DO complex is recognizable by Mags.DO5 conformation specific antibody.**

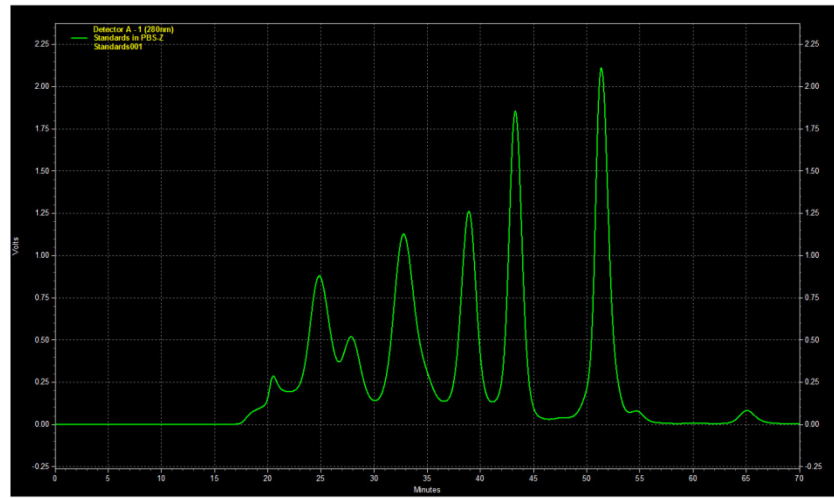
SPR sensograms showing binding of DM/DO (Ni-NTA purified) (green trace), DM (red trace), and DM/DO (Ni-NTA purified) pre-bound to soluble Mags.DO5 (blue trace) to Mags.DO5 antibody coupled chip surfaces.



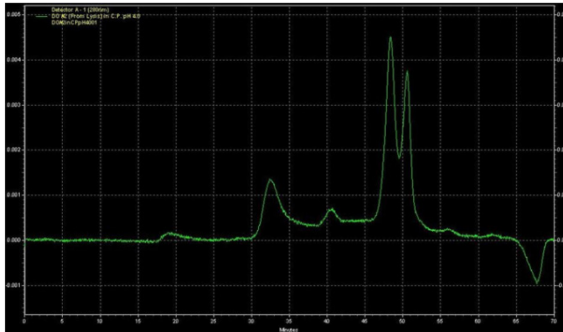
**Figure 5. DO storage conditions affect its function.**

HPLC traces of recombinant DO stored in buffers of varying pH. DO was run through a Superdex™ 200 10/300 GL size exclusion column at 0.2ml/min for 70 minutes. The HPLC mass standards are displayed in the top panel.

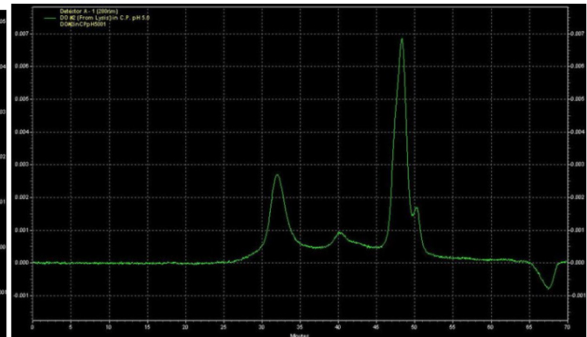
# HPLC Standards



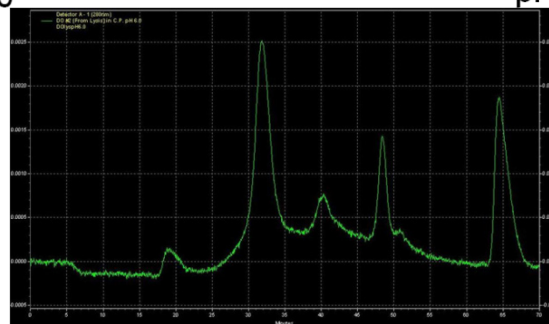
↑ Protein 670KDa  
↑ Aggregates 158KDa  
↑ 44KDa  
↑ 17KDa  
↑ 13.5KDa



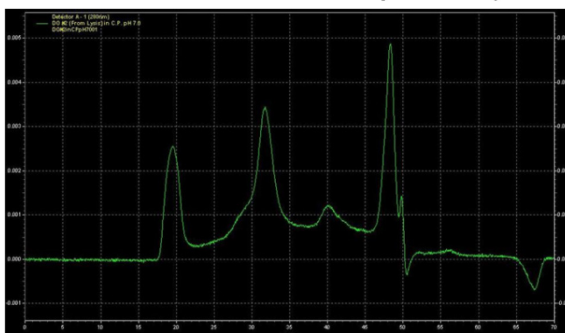
pH 4.0



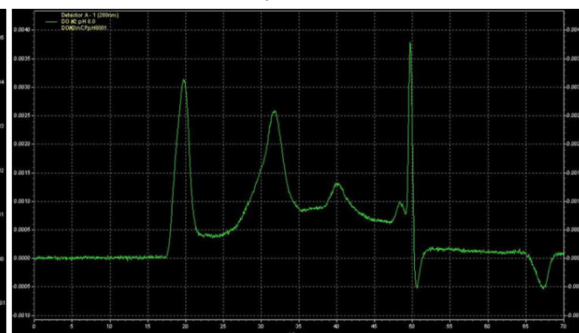
pH 5.0



pH 6.0 (used to store the stock)



pH 7.0



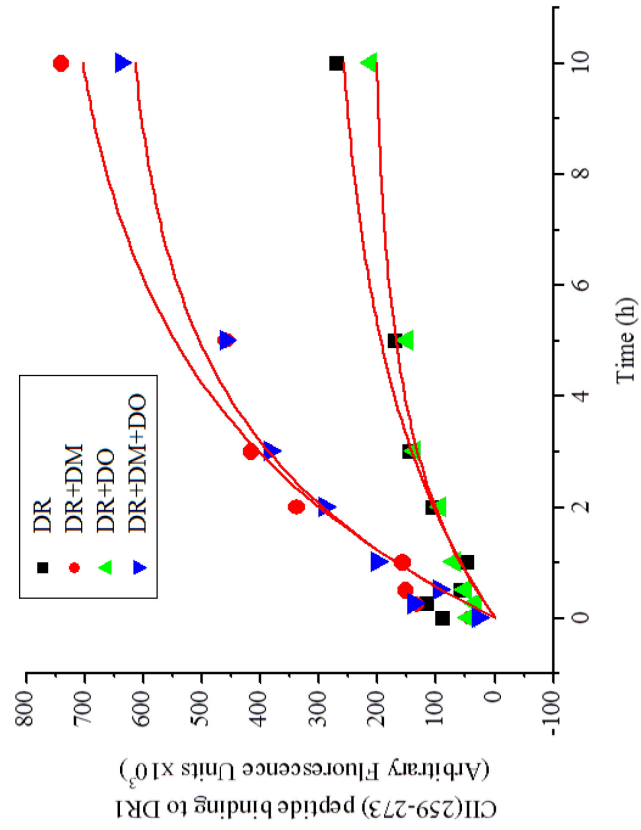
pH 8.0

**Figure 6. Only DO stored at pH 6.0 can have an effect on CII(259-273) peptide binding to DR1.**

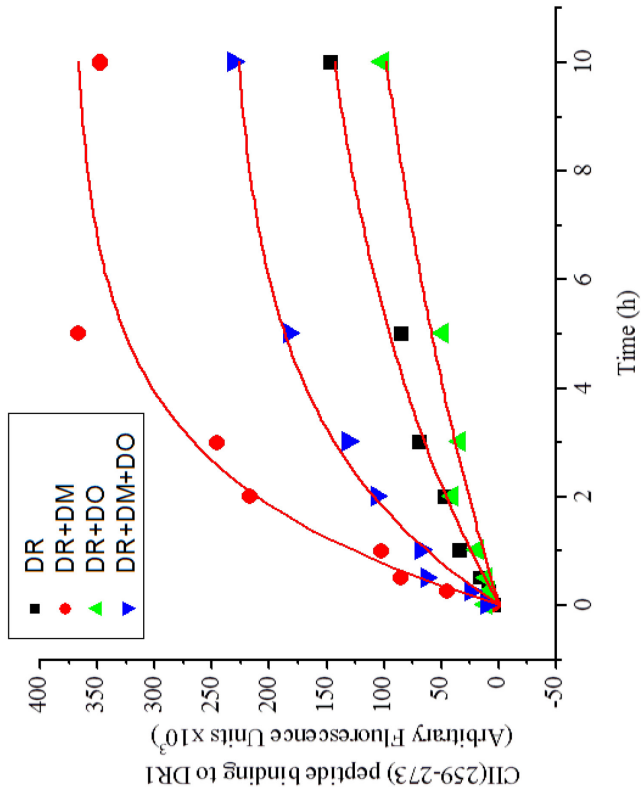
Association of CII(259-273) peptide to DR1 molecules with no accessory molecules (black squares), with DM (red dots), with DO (green triangles), or both DO and DM (blue triangles) over the course of 10 hours. DO used in experiment on the left was stored at pH 7.4 (PBS) while the DO used in the experiment on the right was in Citrate-Phosphate pH 6.0.



**DO stored at pH 7.4:**

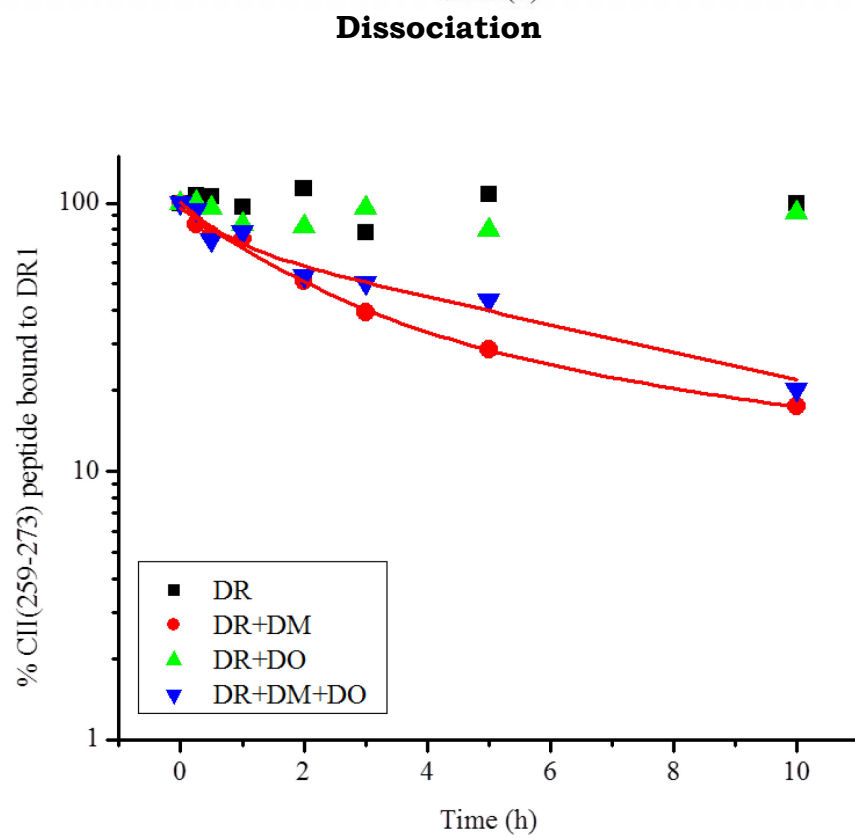
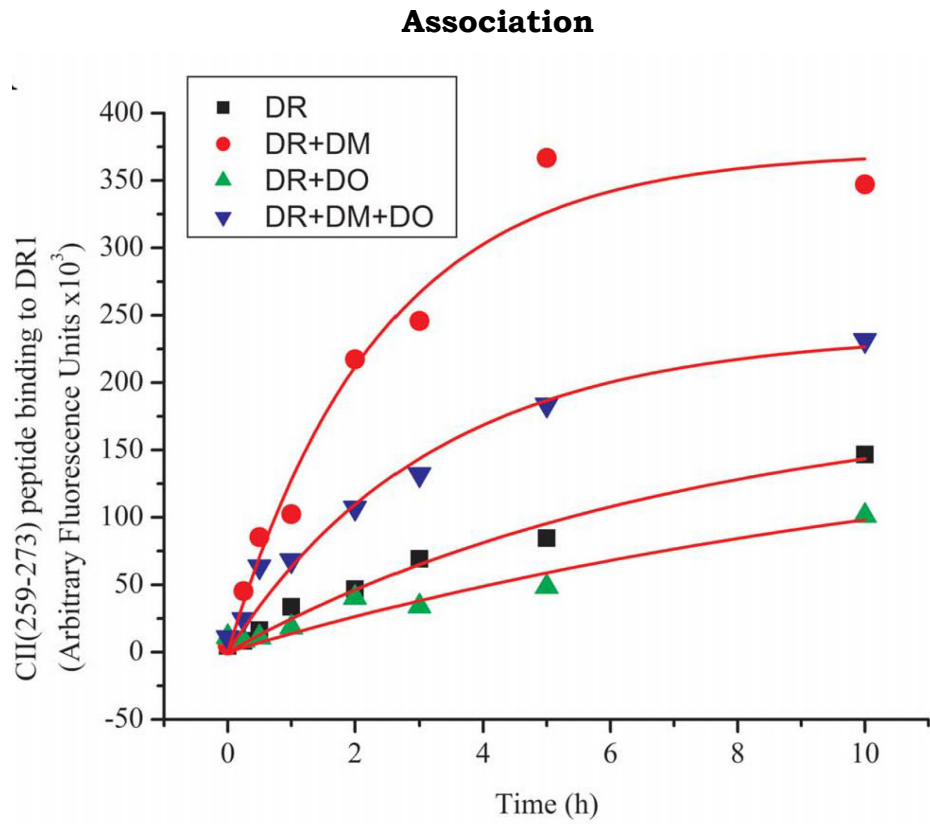


**DO stored at pH 6.0:**



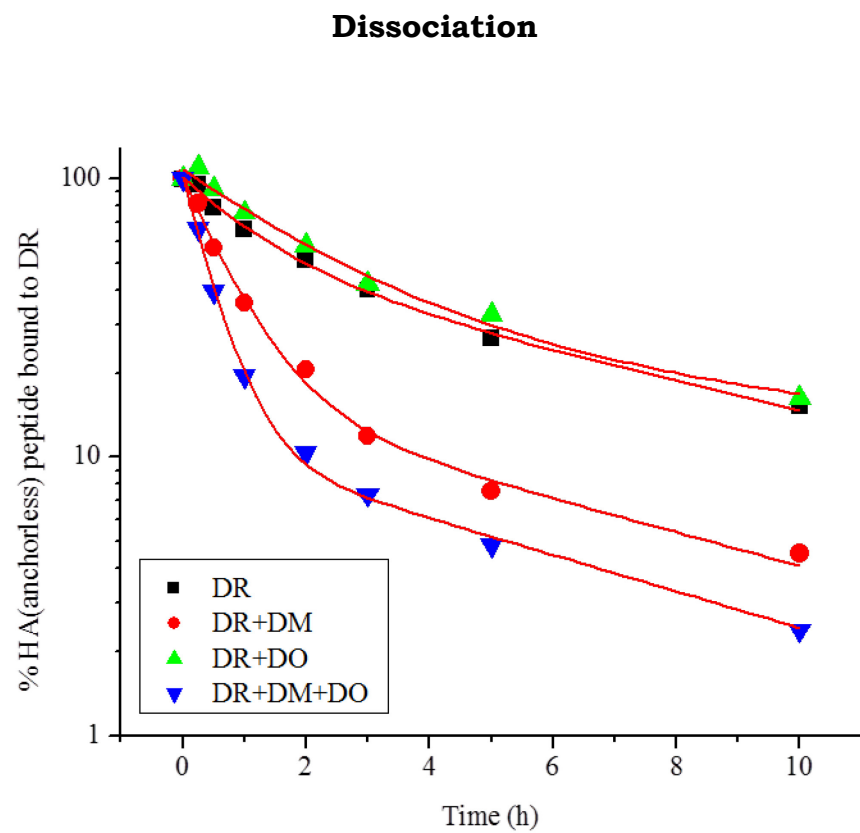
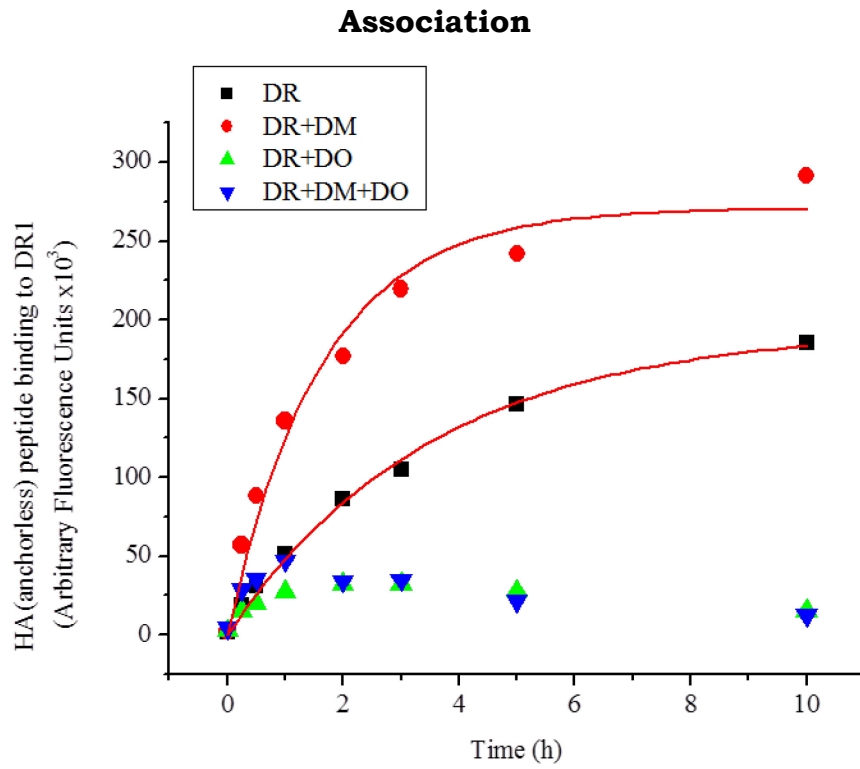
**Figure 7. DO diminishes binding of CII(259-273) peptide to DR1 molecules.**

Association (top) and dissociation (bottom) of CII(259-273) peptide to DR1 molecules with no accessory molecules (black squares), with DM (red dots), with DO (green triangles), or both DO and DM (blue triangles) over the course of 10 hours. The fluorescence signals (Arbitrary Fluorescence Units) associated with the control samples incubated > 10 hours in the absence of DR1 were measured: CII(259-273) peptide alone, 3996; CII(259-273) + DM, 1026; CII(259-273) + DO, 3326; CII(259-273) + DM + DO, 8278.



**Figure 8. DO diminishes binding of HA(anchorless)peptide to DR1 molecules.**

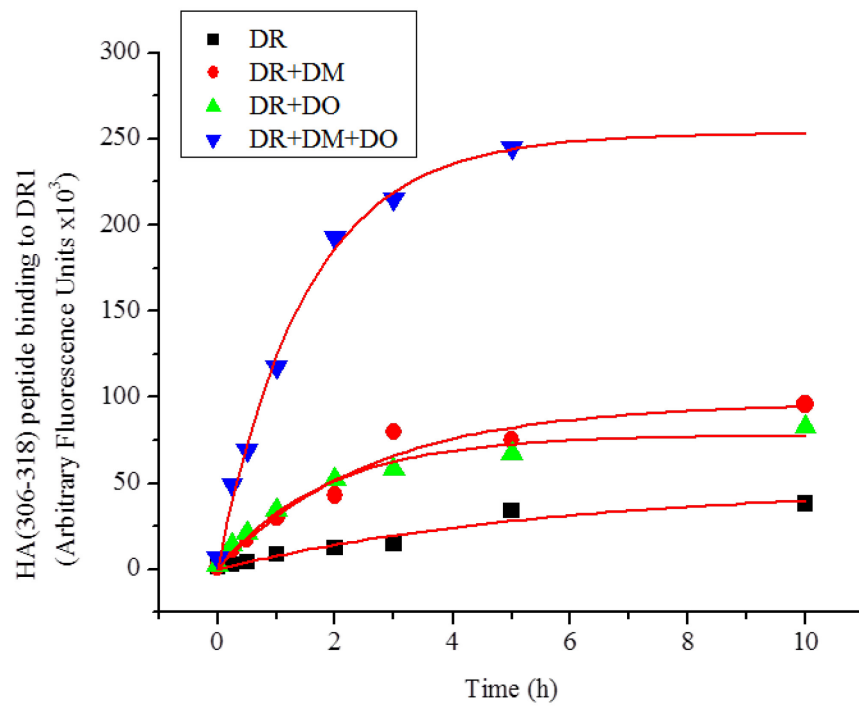
Association (top) and dissociation (bottom) of HA(anchorless) peptide to DR1 molecules with no accessory molecules (black squares), with DM (red dots), DO (green triangles) or both DO and DM (blue triangles) over the course of 10 hours. The fluorescence signals (Arbitrary Fluorescence Units) associated with the control samples incubated > 10 hours in the absence of DR1 were measured: HA(anchorless) peptide alone, 3364; HA(anchorless) + DM, 1334; HA(anchorless) + DO, 1558; HA(anchorless) + DM + DO, 1726. Data shown are representative of at least three independent experiments.



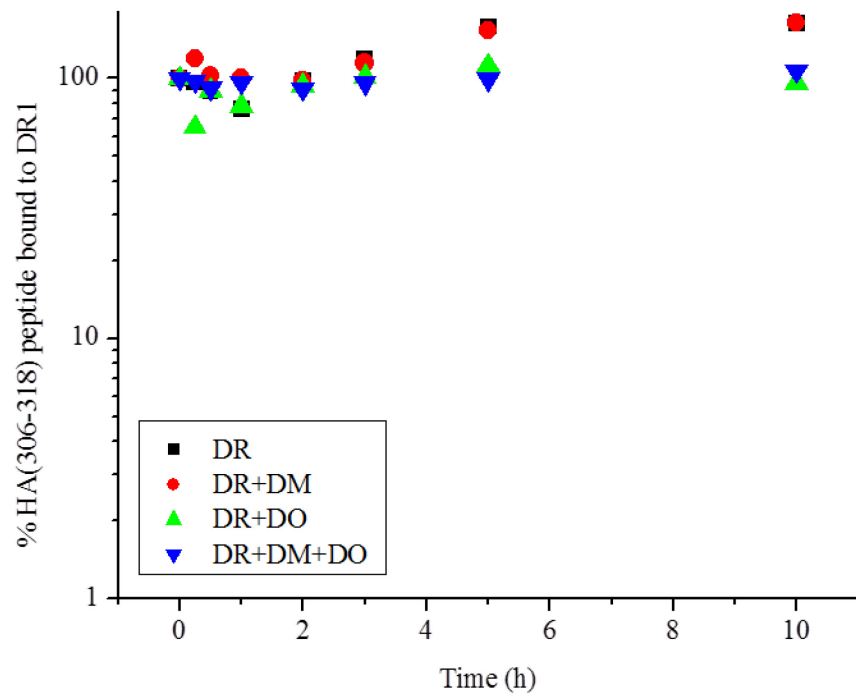
**Figure 9. DO can increase the binding of HA(306-318) peptide to DR1 molecules.**

Association (top) and dissociation (bottom) of HA(306-318) peptide to DR1 molecules with no accessory molecules (black squares), with DM (red dots), DO (green triangles) or both DO and DM (blue triangles) over the course of 10 hours. The fluorescence signals (Arbitrary Fluorescence Units) associated with the control samples incubated > 10 hours in the absence of DR1 were measured: HA(306-318) peptide alone, 1390; HA(306-318) + DM, 1376; HA(306-318) + DO, 3316; HA(306-318) + DM + DO, 9236.

### Association



### Dissociation

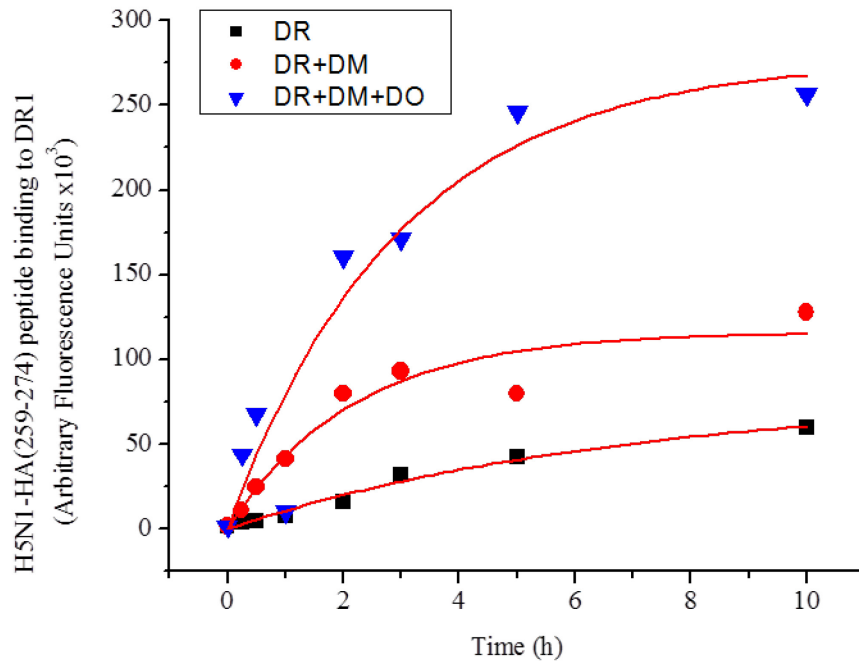


**Figure 10. DO can increase the binding of H5N1-HA1(259-274)peptide to DR1 molecules.**

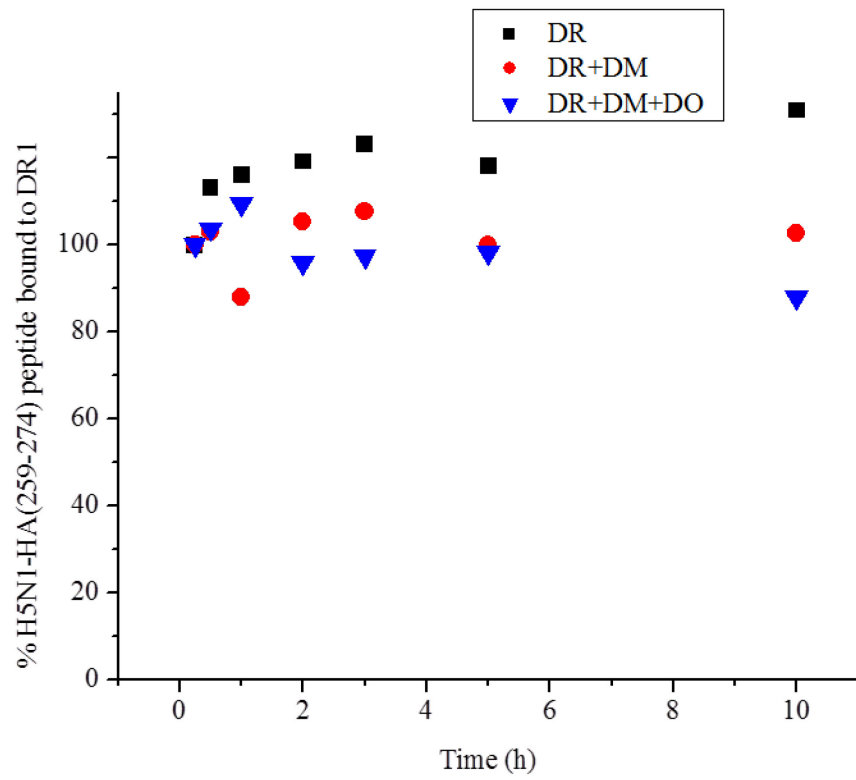
Association (top) and dissociation (bottom) of H5N1-HA1(259-274) flu peptide to DR1 molecules with no accessory molecules (black squares), with DM (red dots), DO (green triangles) or both DO and DM (blue triangles) over the course of 10 hours. The fluorescence signals (Arbitrary Fluorescence Units) associated with the control samples incubated > 10 hours in the absence of DR1 were measured: H5N1-HA1(259-274) peptide alone, 1312; H5N1-HA1(259-274) + DM, 1250; H5N1-HA1(259-274) + DM + DO, 9012.



### Association



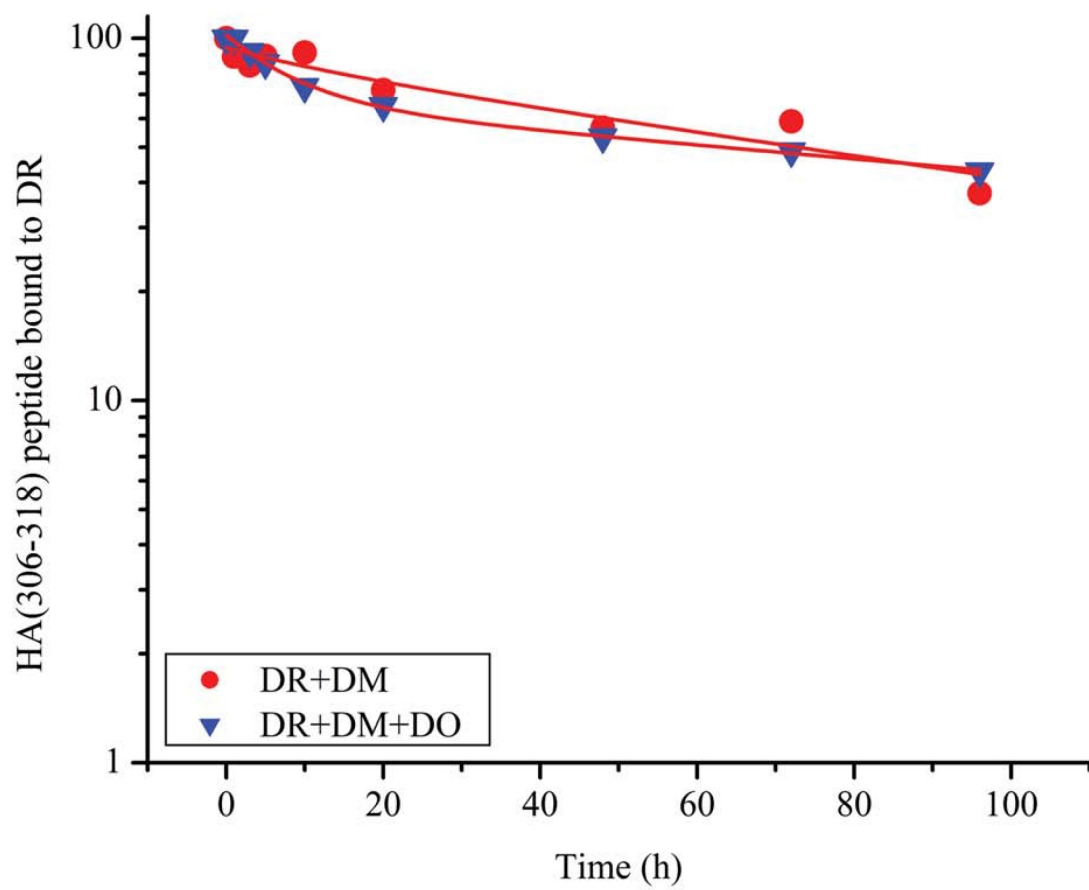
### Dissociation



**Figure 11. DO does not affect the dissociation of HA(306-318) even after 96 hours of incubation.**

Prolonged 96 hour dissociation experiment of HA(306-318) peptide from DR1 molecules with DM (red dots) or both DO and DM (blue triangles).

Data shown are representative of at least three independent experiments.

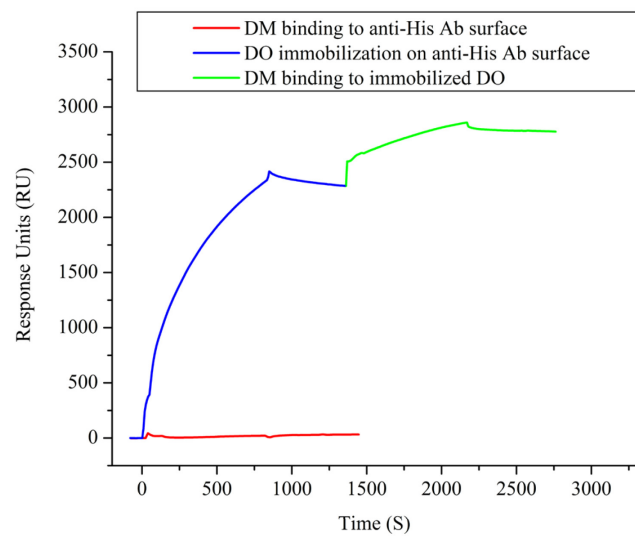
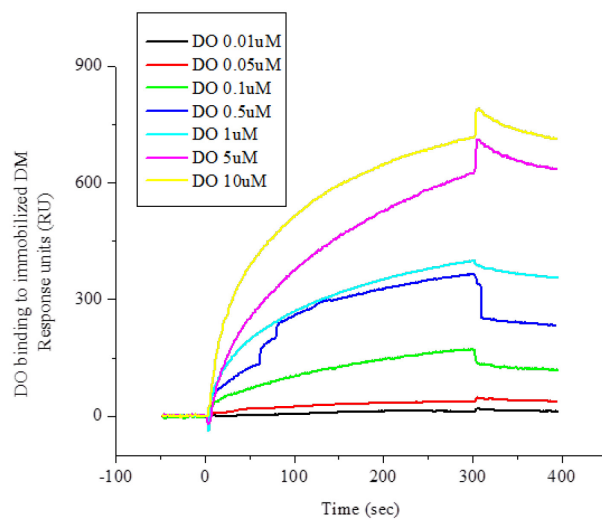
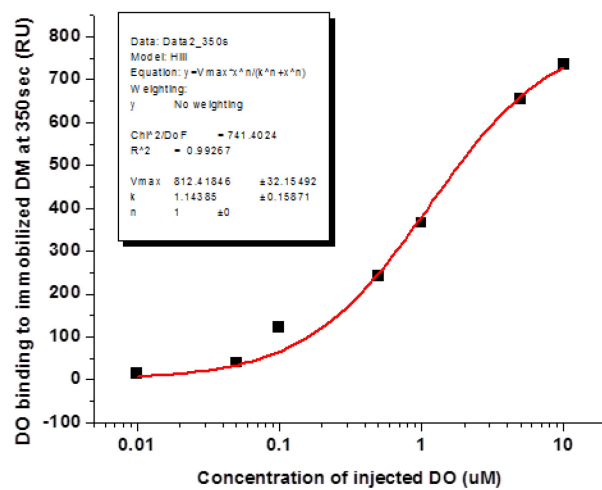


**Figure 12. Soluble recombinant DO forms a complex with soluble recombinant DM.**

(A) DO (Ni-NTA purified) was immobilized on an anti-His antibody surface (blue trace). After a brief wash, DM was injected over the captured DO (green trace). A control injection of DM over anti-His antibody surface (red trace) showed no non-specific binding. Data shown are representative of six independent experiments.

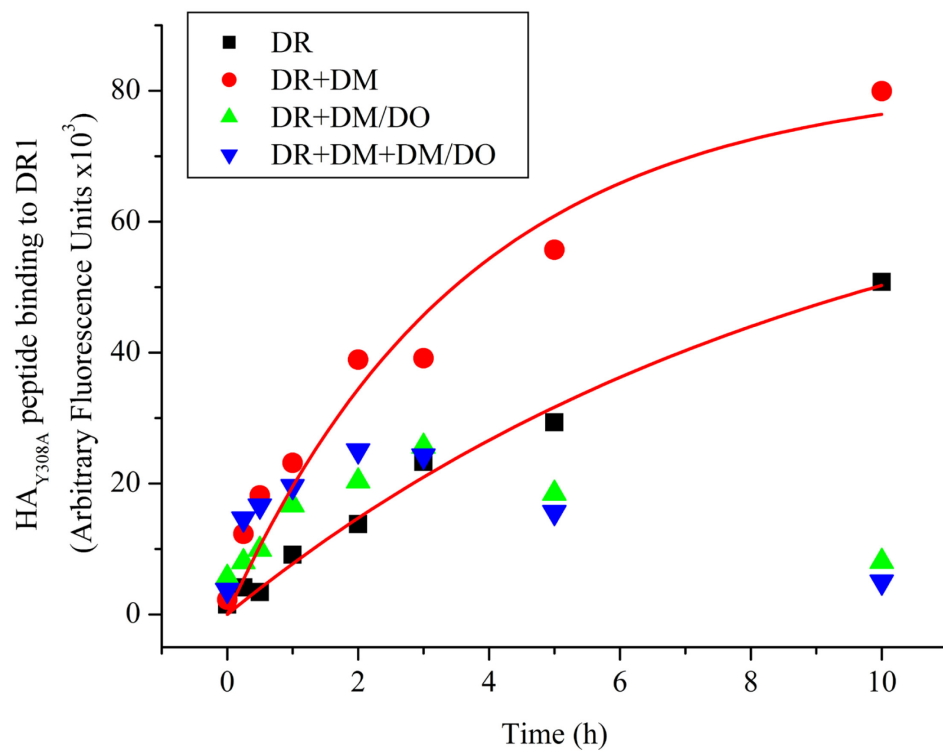
(B) DO (Ni-NTA purified) binding to DM immobilized by anti-FLAG antibody surface in concentrations ranging from 0.01 to 10  $\mu$ M in separate experiments.

(C) Langmuir 1:1 binding isotherm fit of DO bound to DM (RU) versus the concentration of DO. The equation:  $Y = \text{Max} * X / (X + Kd)$ , was used to determine the  $Kd$  of DO binding to DM. Our fit approximated the  $Kd$  to be around 0.5  $\mu$ M.

**A****B****C**

**Figure 13. DO in complex with DM is functionally active.**

Association of HA(Y308A) peptide to DR1 molecules in the presence of co-infected DM/DO complex (Ni-NTA purified). The peptide binding experiment was performed with no accessory molecules (black squares), with DM (red dots), DM/DO (green triangles), or both DM/DO and DM (blue triangles) over the course of 10 hours. The fluorescence signals (Arbitrary Fluorescence Units) associated with the control samples incubated > 10 hours in the absence of DR1 were measured: HA(Y308A) peptide alone, 1140; HA(Y308A) + DM, 894; HA(Y308A) + DM/DO, 1404; HA(Y308A) + DM + DM/DO, 1944.



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## Chapter Three

### **DO acts independently of DM by forming a complex with DR1 when DR1 is in a peptide-receptive conformation**

*\* Parts of this chapter are adapted from a published article co-authored by Yuri Poluektov, titled “HLA-DO as the optimizer of epitope selection for MHC class II antigen presentation” [PLoS One, August, 2013]*

#### **Introduction**

Studies on DO have failed to produce a unified opinion on its role in the MHC II antigen processing system. Initial findings have made a strong case for DM and DO having a linked function, due to DO's reliance on DM for intracellular transport and assembly<sup>1</sup>. Many studies have attributed the effects of DO to the dampening of peptide binding to MHC II molecules through its interaction with DM<sup>2-4</sup>. In contrast, other reports have shown the contrary by identifying DO as an enhancer of peptide presentation<sup>5-7</sup>. For the most part, studies of DO examined complex in vivo systems that made conclusions based on the final outcome of antigen presentation. This makes it hard to develop a mechanism through which DO might interact with DM to diminish or even enhance peptide presentation. In our study, described in Chapter 2, we took a minimalistic approach and examined the direct effect of DO on antigen presentation. We utilized well known techniques established by our and other labs in the field to examine the direct effect of soluble recombinant

DO on peptide association and dissociation from soluble recombinant DR1. This same approach was used in the past to establish the role of CLIP as a place-keeper for the MHC class II groove, inhibiting conformational changes that lead to the inactivation of MHC II before it is able to bind antigenic peptides<sup>8-15</sup>. In addition, this approach laid the foundation for the theory behind the ability of DM to edit the MHC II peptide repertoire by recognizing the heterogeneous conformations of DR1<sup>16-20</sup>. Now we investigate the structural features of the antigenic peptides that have impacts on how DO may regulate their presentation.

The experiments described in Chapter 2 established that DO can have both enhancing and diminishing effects on peptide binding to DR1. To verify the specificity of this effect, we performed a DO depletion experiment where anti-His tag Ab as well as MAGS.DO5 Ab resin were used to deplete DO from the reaction, and observed a decrease in both the enhancing and diminishing effects of DO. The variation in the DO effect seems to correlate with the strength of the anchoring residues of the peptide; specifically, the amino acid that fits into the P1 pocket of the DR1 molecule. It has been shown that the interaction of an antigenic peptide with the P1 pocket of the DR1 molecule is most important in its ability to generate an immunodominant response as well as determining whether DM is able to dissociate it<sup>19</sup>. It is of little surprise that this critical peptide residue would have an effect on DO as well as DM. This

may explain the observed dependence between DM and DO<sup>5,21</sup>. The association of DM sensitive peptides, HA(Y308A) and HA(anchorless), to DR1 in the presence of DO has indicated that the effect of DO is superior to that of DM and that there is a strong possibility that DO acts independently of DM. To validate this, we performed additional kinetic experiments with peptide-receptive DR1 molecules. We hypothesize that not only is DO capable of interacting with DR1 independently of DM, but that it interacts exclusively with DR1 molecules in its receptive conformation.

The result of a peptide-association experiment with the mutant DR1 $\beta$ G86Y molecule, which is permanently stabilized in a peptide-receptive conformation and does not interact with DM<sup>19,22,23</sup>, showed that DO still maintains its ability to inhibit peptide dissociation even when DM is no longer active. This provides strong evidence for DO having a unique function independent of DM. Via SPR, we observed direct binding between DO and peptide-receptive, but not closed or compact, peptide/DR1 complexes. We find that the binding is maintained even when DM/DO complexes are used instead of DO alone, further validating our observation that DO in complex with DM retains its unique function.

Considering all of our data, we have constructed a model for the mechanism of peptide epitope selection on MHC II by DO. We

hypothesize that DO requires DM to generate a peptide-receptive conformation in MHC II molecules, after which DO can bind the receptive molecule and stabilize it in a form that allows for effective binding of long-lived peptides (generally DM-insensitive) and diminishes the binding of short-lived peptides that dissociate from MHC II within hours (DM-sensitive). Thereby DO acts not to inhibit DM but to supplement DM's ability to select for highly stable antigenic peptides to serve as immunodominant epitopes to be presented to T cells. While DM works by dissociating unstable peptides from MHC II, DO prevents the binding of those same peptides once the MHC II molecule is in a peptide-receptive conformation.

## **Materials and methods**

### **A. Production of soluble recombinant MHC class II proteins**

Soluble DR1, DM, DO and mutant DR1 $\beta$ G86Y proteins were expressed and purified as originally described in Chapter 2. Baculovirus DNA (BaculoGold; BD Biosciences, San Jose, CA) and transfer vectors carrying the recombinant soluble forms of the alpha and beta chains of DR1, DM and DO were transfected into SF9 insect cells for recombinant virus production, as suggested by the BaculoGold protocol. Hi5 cells, upon reaching a concentration of  $\approx 1 \times 10^6$  cells/ml were infected with the recombinant virus and grown in suspension in shaker flasks for 3 days



following infection for protein production. Soluble DR1 and mutant DR1 $\beta$ G86Y was purified from both the cells and culture supernatant using anti-DR1 mAb (L243) coupled resin. For the purification of DM and DO, the protocol was modified to use the M2 FLAG (Sigma-Aldrich, St. Louis, MO, USA) and Ni-NTA (QIAGEN, Valencia, CA, USA) affinity columns respectively using gravity flow. Upon elution from the columns DM and DO molecules were concentrated (20-40  $\mu$ M) in Citrate Phosphate pH 6.0 buffer with 0.05% NaN<sub>3</sub> and stored frozen at -80°C in small aliquots. The soluble DR1 heterodimers were kept in PBS pH 7.4 with 0.05% NaN<sub>3</sub>.

## **B. Peptide synthesis and labeling**

See Chapter 2.

## **C. Peptide association and dissociation assays**

See Chapter 2.

## **D. Generation of the peptide-receptive DR1 complex**

Peptide-receptive DR1 was induced by incubating empty DR1 (1-10  $\mu$ M) molecules with excess amount of unlabeled HA(anchorless) peptide (100  $\mu$ M) for three days. Before the start of an experiment which required the use of receptive DR1, generated in the absence of DM, DR1/HA(anchorless) complexes were separated from the excess peptide

by Sephadex G-50 size-exclusion spin columns. Due to the rapid dissociation of HA(anchorless) peptide from DR1, a ( $T_{1/2}$  ~90 min), a significant number of DR1 molecules convert to a DR-receptive state within a short time<sup>14</sup>. Any long-lived peptide would displace HA(anchorless) and bind to DR1 molecules as if they were receptive.

#### **E. DO depletion through immunoprecipitation**

DO was depleted from our DO containing samples by tandem incubations with Ni-NTA and Mags.DO5 antibody resins. First DO (4  $\mu$ M) was added to 20-25  $\mu$ l of 50 % slurry of Ni-NTA resin and incubated for 20 minutes. The resin was then spun in a table top centrifuge and the supernatant was collected and added to 20-25  $\mu$ l of 50 % slurry of Mags.DO5 resin. After another 20 minutes of incubation the Mags.DO5 resin was spun and the supernatant containing the depleted DO was added directly to the binding reaction.

#### **F. Surface Plasmon Resonance measurements of protein binding**

SPR experiments were performed as described previously on a BIAcore 1000 instrument (GE Healthcare)<sup>24</sup>. Anti-His tag (Invitrogen) antibody was coupled to CM5 chips (GE Healthcare) through standard amine coupling procedures. Excess activated dextran carboxylate groups were capped with 2-amino-ethyl-sulfate (pH 8.0) to decrease the nonspecific binding of MHC molecules to the chip surface at a lower pH<sup>25</sup>. DO or DM/DO

complexes (4  $\mu$ M) were immobilized on top of the anti-His tag antibody. DR1 receptive or closed and mutant DR1 $\beta$ G86Y (4  $\mu$ M) were then tested for binding to DO. All protein solutions were diluted in the running buffer composed of Citrate-Phosphate pH 5.5 with 150 mM NaCl, 0.005% Tween-20 and 0.05% NaN<sub>3</sub>. Measurements were taken at 27°C with flow rates ranging between 5-10  $\mu$ l/min. The magnitude of binding was measured at the *stability point* ~200-300 seconds after the end of the injection.

### **Data analysis**

See Chapter 2.

## **Results**

### **A. The effect of DO on peptide binding to DR1 is the direct result of DO**

As a validation of our peptide association and dissociation experiments that showed DO could have both an enhancing and a diminishing effect on peptide binding to DR1, we tested the specificity of this effect. For this purpose DO was depleted from the sample by tandem runs immunoprecipitation (IP) over anti-His antibody and a conformation-specific antibody (Mags.DO5) resin (see Materials and Methods). The sample of DO depleted as such was compared to the samples with and

without DO for their ability to diminish the binding of HA(anchorless) peptide (**Fig.1A**) or enhance the binding of HA(306-318) peptide (**Fig.1B**) after a 5 hour incubation with DR1. In both cases the depletion of DO resulted in a decrease in the diminution of the binding of HA(anchorless) (**Fig.1A 2<sup>nd</sup> and 5<sup>th</sup> bars**) and the enhancing effect on the binding of HA(306-318) (**Fig.1B 2<sup>nd</sup> and 5<sup>th</sup> bars**). Both experiments were repeated in the presence of DM, and in each case the depletion of DO through IP decreased its effect on the binding of peptides. This strongly suggests that both the decrease and increase in binding of peptides can be attributed to the DO molecule and not any other protein.

#### **B. DO acts directly on DR1 rather than by modifying DM**

Peptide association experiments described in Chapter 2 demonstrated that for HA(anchorless) and HA(Y308A) peptides DO plays an overwhelming role even in the presence of DM. If DO were to inhibit DM, the decrease in peptide binding to DR1 in the presence of DO would have been different when both DM and DO were present. In both cases however, the decrease in binding induced by DO was the same, strongly suggesting that DO acts directly on DR. To test this idea we examined the effect of DO on DR1 molecules.

Because DO did not affect dissociation of peptide/DR complexes, a process that DM does best, we hypothesized that perhaps DO can only interact with a receptive DR1. To test this idea, peptide-receptive DR1 was generated by incubating DR1 molecules with HA(anchorless) peptide and separating the peptide/DR1 complexes from excess peptide and then allowing DR1 to incubate at 37 °C for a few minutes (see materials and methods). Because of short half-life of HA(anchorless)/DR1 complexes a peptide-receptive conformation would be generated when the peptide dissociates from DR1<sup>19</sup>. As such, we are able to monitor the effect of DO on the binding of HA(306-318) (**Fig.2A**) and CII(259-273) (**Fig.2B**) to receptive DR1 molecules. While no DM was present in the system, DO was still able to enhance the binding of HA(306-318) and slightly diminish the binding of CII(259-273) to DR1, as was observed in Chapter 2. The absence of DM did not prevent DO from affecting peptide association to DR1, as long as the peptide-receptive conformation was induced.

### **C. DO is able to interact with a mutant DR1 molecule constitutively in a peptide-receptive conformation**

To further evaluate the idea that DO interacts with a peptide-receptive conformation of DR1, we examined the effect of DO on a mutant DR1 $\beta$ G86Y molecule. This mutant DR1 has a filled P1 pocket which forces it to remain in a peptide-receptive conformation, unable to shift to

a closed compact form<sup>19,22,23</sup>. In addition, this mutation prevents the DR1 molecule from binding most long-lived peptides with bulky residues destined for the P1 pocket. If the effect of DO is similar to that of DM then it should not be detectable with mutant DR1 $\beta$ G86Y, since DM enhances the binding of peptides by forcing the MHC II molecules to adopt a peptide-receptive conformation.

We monitored association kinetics of HA(anchorless) peptide to mutant DR1 $\beta$ G86Y over the course of 10 hours (**Fig. 3A**). To our surprise, despite the inability of DM to enhance peptide binding to DR1 $\beta$ G86Y, DO could still perform its function of diminishing the binding of HA(anchorless), as previously observed in Chapter 2. This suggests that DO does not participate in the generation of peptide-receptive DR1 molecules.

#### **D. DO forms a stable complex with mutant DR1 $\beta$ G86Y molecules but not compact closed DR1**

All of our observations point towards DO interacting with receptive DR1 molecules. If this is indeed the case, there is a possibility we might be able to detect a complex formation between DO and receptive DR1 using the SPR technique. The ability of SPR to detect the binding of MHC II molecules has been previously demonstrated in a study that observed the interactions of DM and DR1<sup>20</sup>. If the DO/DR1 complex is stable enough we would be able to visualize the binding between the two molecules in our BIAcore 1000 instrument. For this purpose we captured

DO on anti-His antibody immobilized CM5 chip followed by the injection of mutant DR1 $\beta$ G86Y molecules (**Fig. 4**). Approximately 100 RU was detected upon completion of DO injection indicating that complexes were formed between DO and DR1 $\beta$ G86Y (**Fig. 4 inset**). In a control experiment when closed compact DR1/HA(306-318) was injected over immobilized DO, the detected binding was 20 RU (**Fig. 5**). A 20 RU binding corresponds to our background level of interaction between DR1 and the anti-His antibody immobilized surface (**Fig. 4-5**).

Our data from Chapter 2 suggests that DM/DO complexes act like DO alone. To explore this observation further, we tested the ability of DM/DO complexes to bind to DR1 $\beta$ G86Y. DM/DO molecules were captured by immobilized anti-His antibody surface to a total signal of 2000-3000 RUs. Then, DR1 $\beta$ G86Y molecules were injected in three concentrations of 0.5, 1 and 2  $\mu$ M over the DM/DO captured surface (**Fig. 6A**). Repeat injections of increasing concentrations of DR1 $\beta$ G86Y were performed on the same flow cell following the prolonged dissociation of the bound DR molecules (0.5  $\mu$ M) or a regeneration of the surface with the injection of pH 11.5 CAPS buffer, washing the surface overnight in running buffer and recapture of 2000-3000 RUs of DM/DO molecules before the next injection (1  $\mu$ M). With increasing concentration of DR1 $\beta$ G86Y more binding was measured when the sensogram stabilized following the end of the injection. Binding controls were included to rule

out any nonspecific binding. Neither DR1 $\beta$ G86Y at 4  $\mu$ M when injected over anti-His antibody coupled surface, nor 4  $\mu$ M of pre-formed HA(306-318)/DR1 complexes (closed conformer) injected over the DM/DO bound surface produced noticeable binding. In order to make sure that the observed binding was specific to DO and not DM in complex with DO, DR1 $\beta$ G86Y and DR1-HA molecules were injected over DM molecules captured by an anti-FLAG antibody coupled chip surface (**Fig. 6B**). The binding of HA(306-318)/DR1 molecules to DM remained at a minimal level of 20 RU, whereas DR1 $\beta$ G86Y binding to DM resulted in 35 RU. In each case the signals were significantly smaller than those resulting from DR1 $\beta$ G86Y binding to DM/DO complexes at lower concentrations.

While the mutant DR1 $\beta$ G86Y molecule is always receptive, wild-type DR1 utilizes the help of DM to adopt a receptive conformation. In a full-fledged MHC II system DM most likely creates the peptide-receptive conformation necessary for the function of DO. To test this, a transient receptive conformation was generated in DR1 molecules by pre-loading them with DM-sensitive HA(Y308A) peptide for 3 days at 37°C (HA(Y308A)/DR). Before injection into the BIAcore instrument, excess HA(Y308A) peptide was removed through G50 column filtration. DM (2 $\mu$ M) was then added to the HA(Y308A)/DR complexes as they were incubated at 37°C for 20 minutes. Due to the short half-time of dissociation of HA-Y308A and the addition of DM, most of the peptide



would dissociate from DR1 molecules leaving them in an open peptide-receptive conformation. Immediately after the 20 minute incubation this solution, which contained peptide-receptive DR1 molecules, DM, and the unbound HA(Y308A) peptide was injected over DM/DO captured surface. By slowly injecting DR1 molecules at a rate of 1  $\mu\text{l}/\text{min}$  for 50 minutes we saw a 337 RU binding of DR1-receptive to DM/DO. Since the receptive conformation is transient and may not be adopted by all the DR1 molecules, unlike with DR1 $\beta$ G86Y, the injection had to be continued over a prolonged period of time while keeping the temperature of the flow-cell at 37°C to allow further generation of receptive DR1 during the injection (**Fig. 7**). Control injections of 4  $\mu\text{M}$  HA(306-318)/DR1 closed conformer under the same conditions produced only 46 RU binding. Because DR-receptive molecules included DM, we also injected DM (2  $\mu\text{M}$ ) as control, which resulted in 228 RU of binding. We believe this rather high level of DM binding to DM/DO surface is due to dissociation of DM from the anti-His captured DM/DO complexes over long incubation at 37°C.

## **Discussion**

Crystallization of the DM/DO complex has led to a model in which upon binding to DO, DM is no longer able to function properly<sup>26</sup>. This, however, does not prevent DO from functioning as an accessory molecule as we have seen in Chapter 2, figure 13. Moreover, the interaction between DM

and DO does not inhibit the complex formation between DM/DO and receptive DR1 molecules depicted in figures 6-7. As we have seen from our data, as well as past experiments, DO forms a stable complex with DM<sup>1</sup>. Although the true proportion of free versus DO-bound DM has not been established, it is generally believed that free DM is present even in DO expressing cells<sup>27</sup>. Our data suggests that both DO and DM/DO complexes will only interact with receptive DR1 molecules. Since one of the main functions of DM is to stabilize this receptive conformation, we hypothesize that the initial action by DM is necessary for DO to perform its function properly. This means that great care has to be given when constructing an experimental system to determine the effects of DO, especially when it comes to transfected cells. If the concentration of DO becomes too high, the effects of DO would be hard to detect, since the system would be missing DM necessary to generate peptide-receptive MHC II molecules that DO can act upon. This may have been one of the reasons behind the difficulty in studying DO over the years.

Once DO or DM/DO interacts with receptive DR1 molecules it forces another conformational change to occur. It is unlikely that DO simply stabilizes the peptide-receptive conformation, since a receptive conformation binds all peptides equally regardless of how long- or short-lived they are. DO, on the other hand, promotes the binding of long-lived DM-insensitive peptides while diminishing or even completely inhibiting

the binding of short-lived DM-sensitive peptides. In order to explain this selection process we like to visualize this new conformation as one that has the DR1 peptide binding groove open beyond the receptive state (super-receptive). In this new conformation the access to the P1 pocket of the DR1 molecule is more readily available, perhaps even the P1 pocket itself is wider than in a receptive state. This allows long-lived peptides, which generally have a big bulky hydrophobic residue dedicated for the P1 pocket, to interact with the P1 pocket more readily than in a receptive DR1. This would hold true for short-lived peptides as well. But short-lived peptides while binding more readily to the P1 pocket would also be more prone to dissociation. In the end this would mean that long-lived peptides remain bound after the action of DO, while short-lived peptides would never get a chance to stabilize themselves in the P1 pocket. Putting our results together we are able to propose a model for the mechanism of DO (**Fig. 8**). We propose that the function of DO is dependent on DM generating a peptide-receptive MHC II molecule. Once a peptide-receptive MHC is available, DO binds to it to induce a conformation different from that of a receptive MHC II. This facilitates the binding of long-lived peptides while diminishing the binding of short-lived peptides. Instead of acting as an inhibitor or an enhancer of DM, DO has its own mode of selection of antigenic peptide epitopes. DO acts as a secondary editor of the MHC II peptide repertoire which narrows down the antigen repertoire even further than DM alone.

We have unfortunately not been able to conduct a successful experiment to prove the existence of a conformation of DR1 different from receptive and closed or compact. We purposed that a DR1 molecule interacting with DO would be less stable than empty DR1 alone, due to the super-receptive conformation it adopts. To test this proposal we tried to determine the melting point of DR1 alone and in the presence of DO through circular dichroism (CD) studies. Our CD experiments were not sensitive enough and produced melting points that were only a few degrees apart, with the DR1 sample in the presence of DO being more stable than that of DR1 alone (**Appendix i**).

In a fully functional class II antigen presentation system, we believe that DO imposes its own level of control on the structure of the peptides selected to be presented to T cells by MHC II molecules. Our proposed mechanism for DO implies that the first step of narrowing down the peptide repertoire occurs by DM dissociating any unwanted peptides from MHC II molecules and leaving them receptive to bind other peptides. DO would then select the peptides that should and should not bind to the MHC II peptide groove. This effectively means that DO would have the final say as to what peptides are presented by MHC II molecules and not DM. Although it is not certain to what extent DO would select from the available peptide pool generated by DM. This raises many

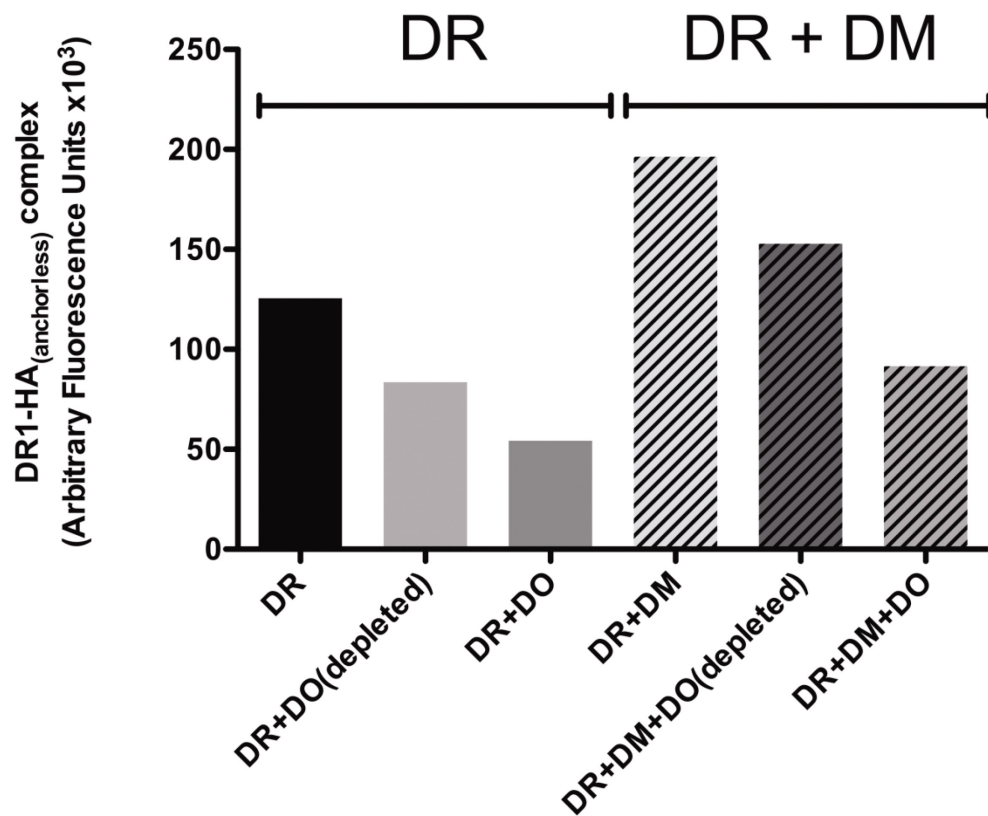
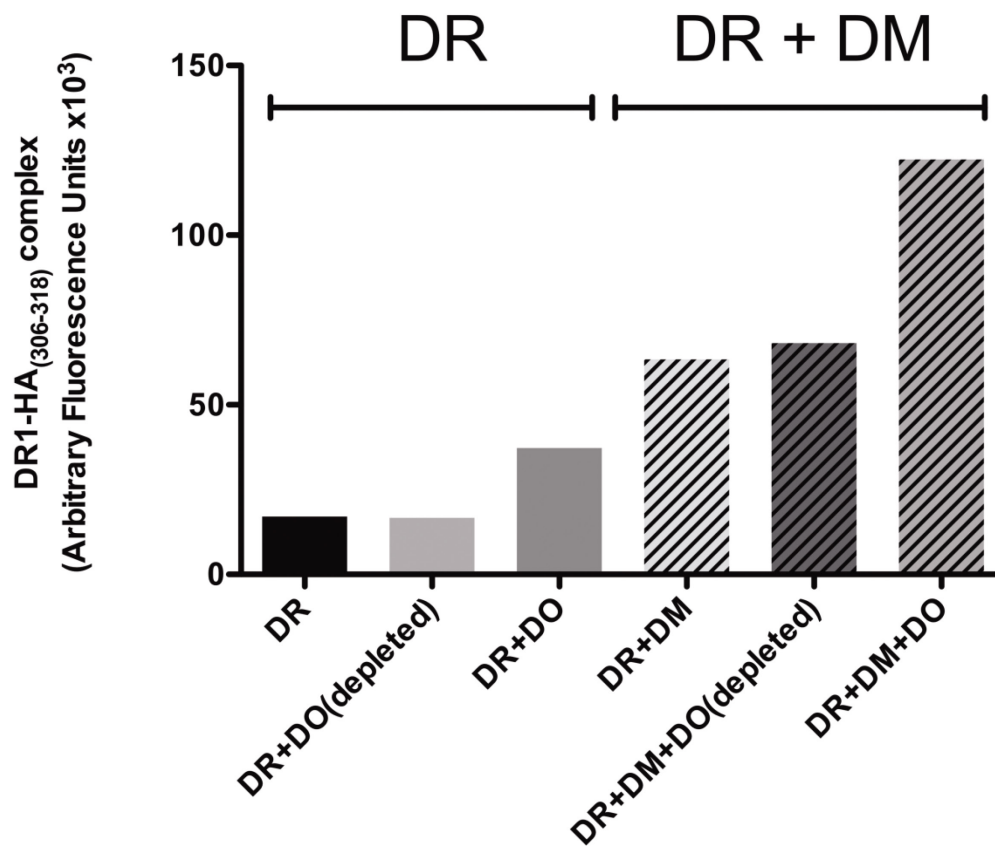
questions about the significance of this new accessory molecule for our immune system.

## Figures

### **Figure 1. The effect of DO can be diminished by DO depletion through immune precipitation.**

(A) DO was depleted from a DO stock by immunoprecipitation via Ni-NTA followed by Mags.DO5 resin. The depleted sample was used instead of DO in reactions measuring HA(anchorless) peptide/DR complex formation in the presence or absence of DM after 5 hours of incubation. The fluorescence intensity of peptide/DR1 complexes formed in the DO depleted reaction was compared to a reaction containing no DO (left bar in each set of three), and a reaction that contained DO that did not undergo depletion (right bar in each set of three). The experiment is representative of three separate trials.

(B) A DO-depleted sample was used instead of DO in a reaction measuring of HA(306-318) peptide/DR complex formation in the presence or absence of DM after 5 hours of incubation. The fluorescence intensity of peptide/DR1 complexes formed in the DO depleted reaction was compared to a reaction containing no DO (left bar in each set of three), and a reaction that contained DO that did not undergo depletion (right bar in each set of three). The experiment is representative of three separate trials.

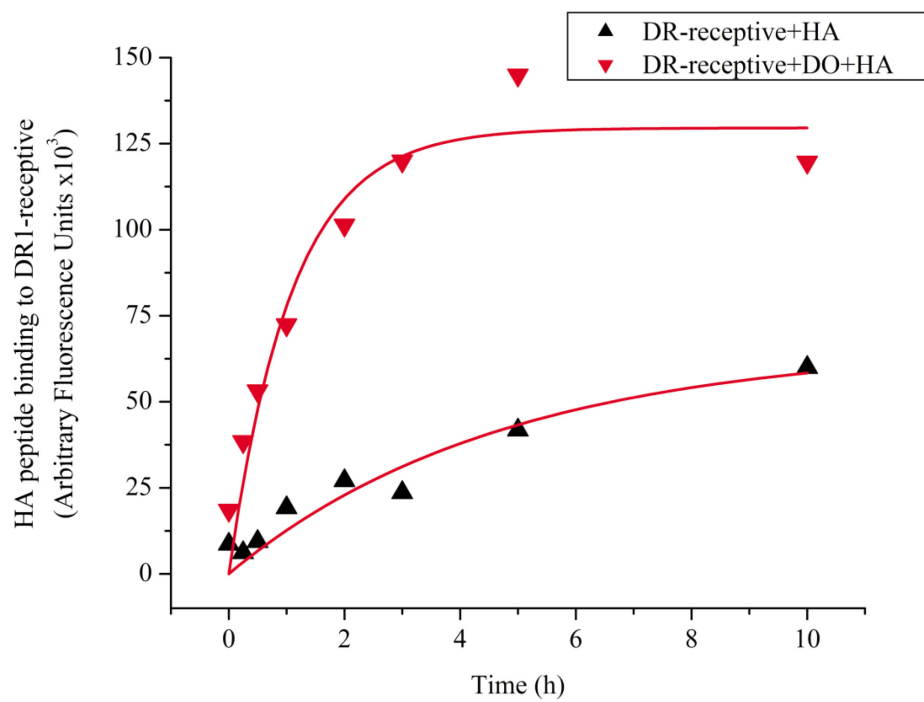
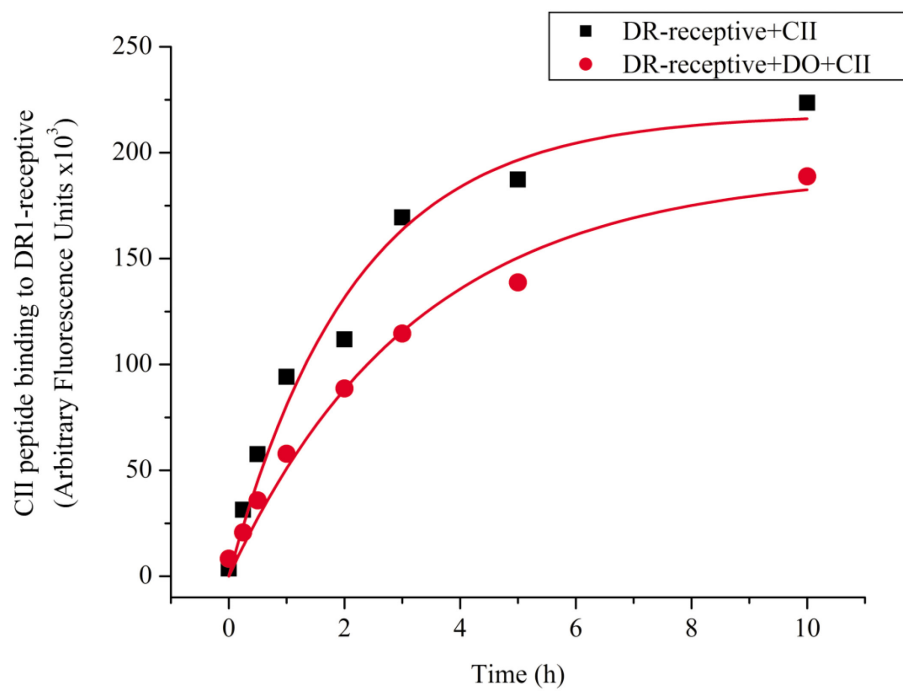
**A****B**

**Figure 2. DO affects peptide binding to DR1 independent of DM.**

(A) Association kinetics of HA(306-318) to peptide-receptive DR1 without DO (black triangles) or with DO (red triangles).

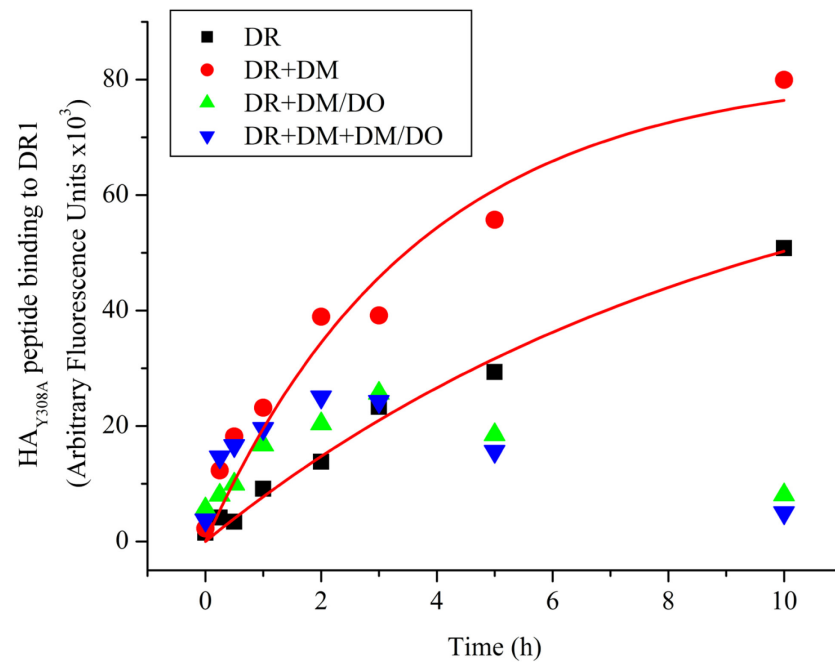
(B) Association kinetics of CII(259-273) to peptide-receptive DR1 without DO (black triangles) or with DO (red triangles).



**A****B**

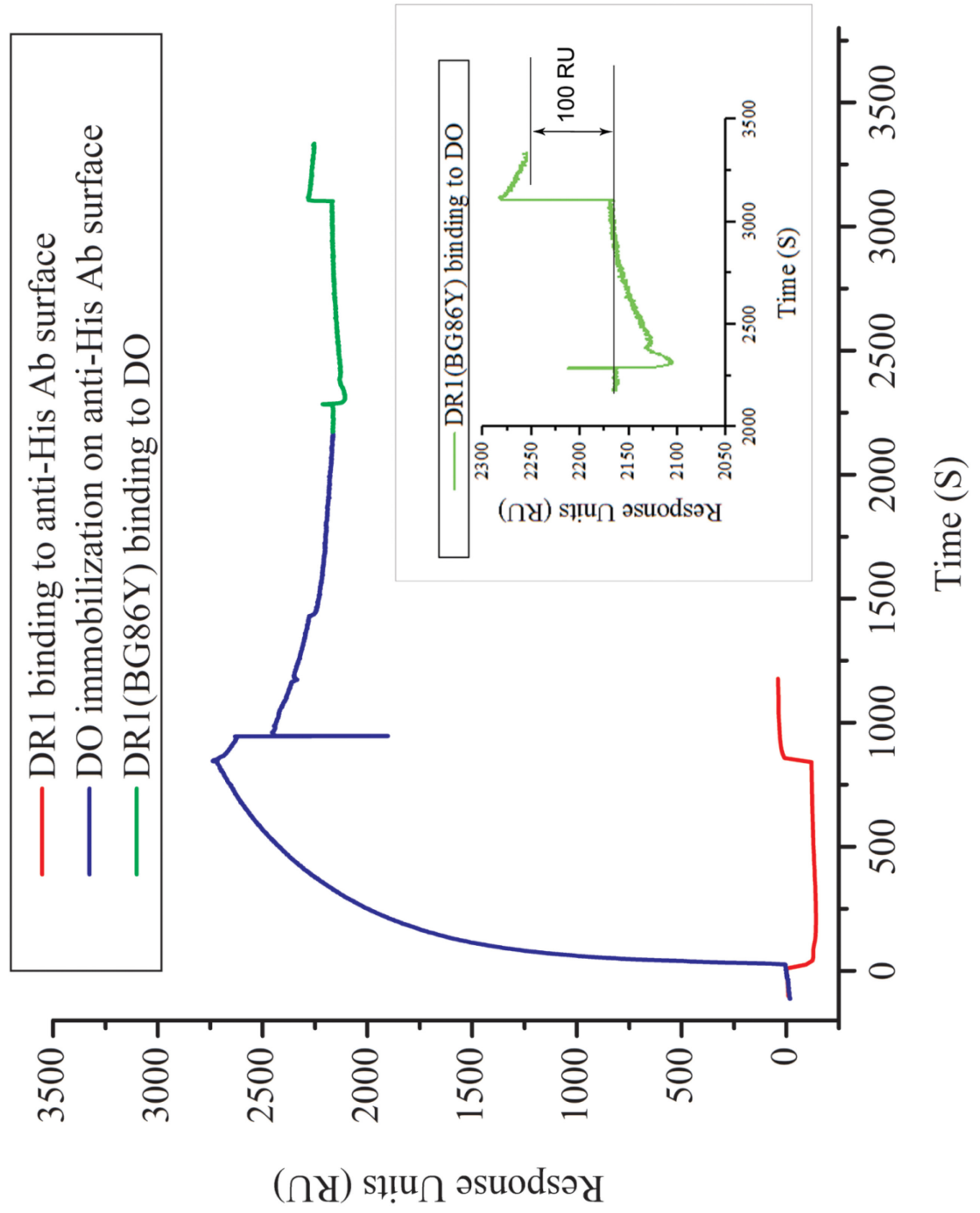
**Figure 3. DO acts upon constitutively receptive DR1 $\beta$ G86Y molecules.**

(A) HA(anchorless) association to constitutively receptive mutant DR1 $\beta$ G86Y molecules with no accessory molecules (black squares), with DM (red dots), DO (green triangles) or both DO and DM (blue triangles). The fluorescence signals (Arbitrary Fluorescence Units) associated with the control samples incubated > 10 hours in the absence of DR1 were measured: HA(anchorless) peptide alone, 1804; HA(anchorless) + DM, 1512; HA(anchorless) + DO, 2280; HA(anchorless) + DM + DO, 2888.

**A**

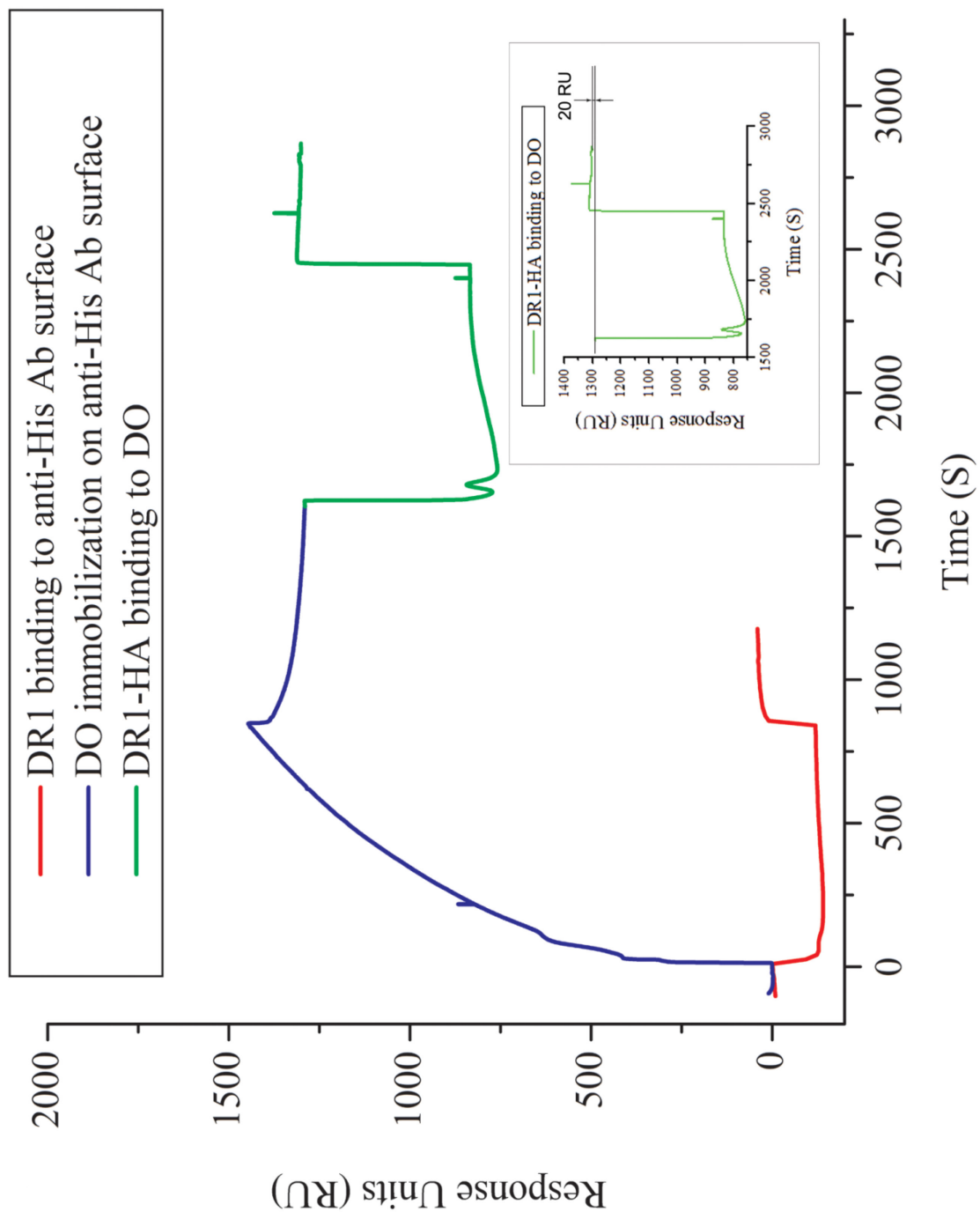
**Figure 4. DO forms a complex with DR1 in a receptive conformation.**

SPR sensograms of constitutively receptive DR1 $\beta$ G86Y (4 $\mu$ M) binding to DO. Ni-NTA purified DO was immobilized on anti-His antibody coupled chip (blue trace). After a brief wash, DR1 $\beta$ G86Y was injected over the captured DO surface (green trace, enlarged in inset). An injection of unloaded DR1 over the anti-His antibody surface (red trace) was performed to control for potential nonspecific binding DR1 to the chip surface.



**Figure 5. DO does not form a complex with DR1 in a compact closed conformation.**

SPR sensograms of closed compact DR1/HA(306-318) complex (4 $\mu$ M) binding to DO. Ni-NTA purified DO was immobilized on anti-His antibody coupled chip (blue trace). After a brief wash, DR1/HA(306-318) was injected over the captured DO surface (green trace, enlarged in inset). An injection of unloaded DR1 over the anti-His antibody surface (red trace) was performed to control for potential nonspecific binding DR1 to the chip surface.

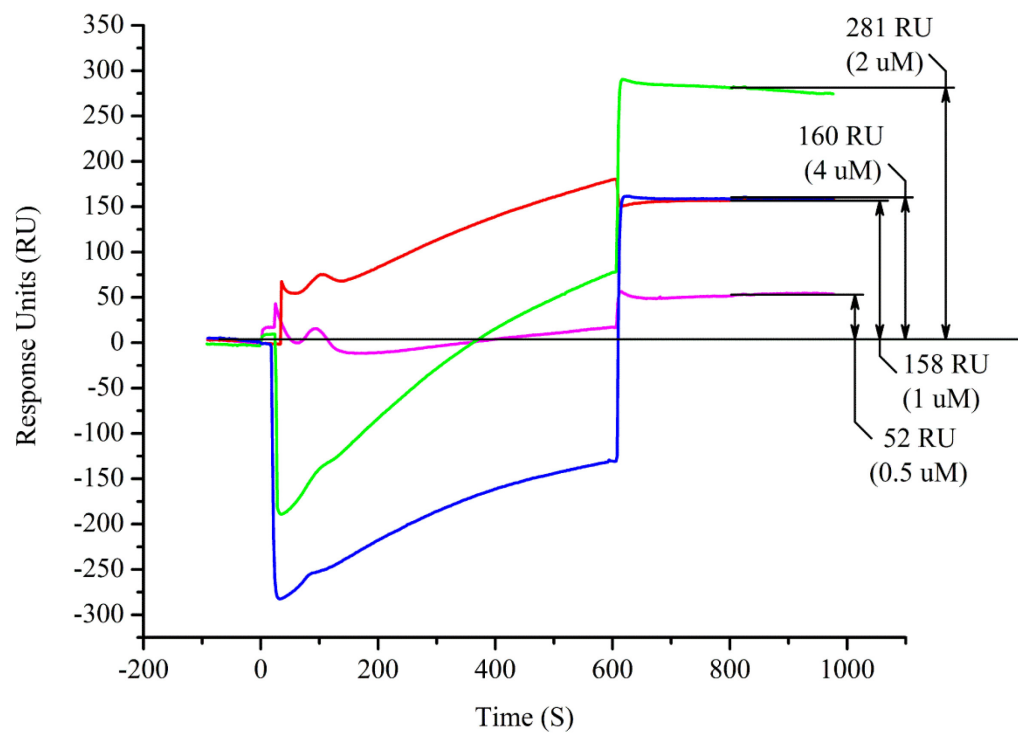
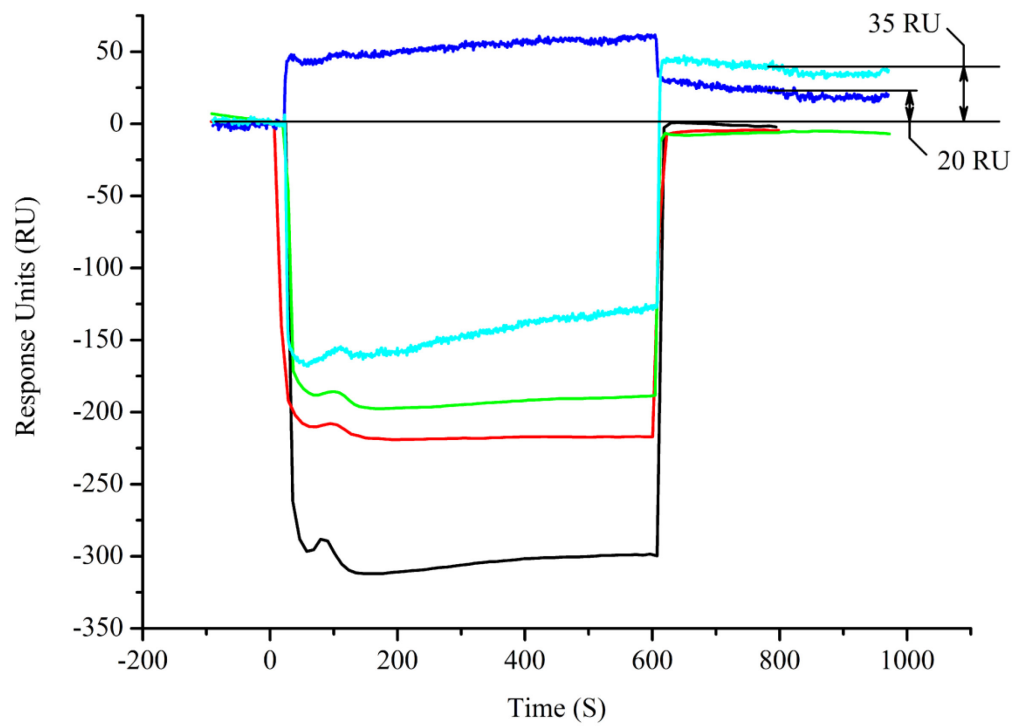


**Figure 6. DM/DO forms a complex with peptide-receptive DR1 molecules.**

(A) DR1 $\beta$ G86Y binding to DM/DO complex molecules. Mags.DO5 purified DM/DO was immobilized on anti-His antibody coupled chip surface to a level of 2000-3000 RU. After a brief wash, DR1 $\beta$ G86Y was injected over the captured DM/DO at concentrations of 0.5  $\mu$ M (blue trace), 1  $\mu$ M (red trace), 2  $\mu$ M (green trace). Before every injection of DR1 $\beta$ G86Y, the DM/DO molecules captured on the surface were regenerated to insure that the surface was not saturated by bound DR1 molecules. The signal of the resulting binding  $\sim$ 200-300 after the end of the injection is marked on the graph.

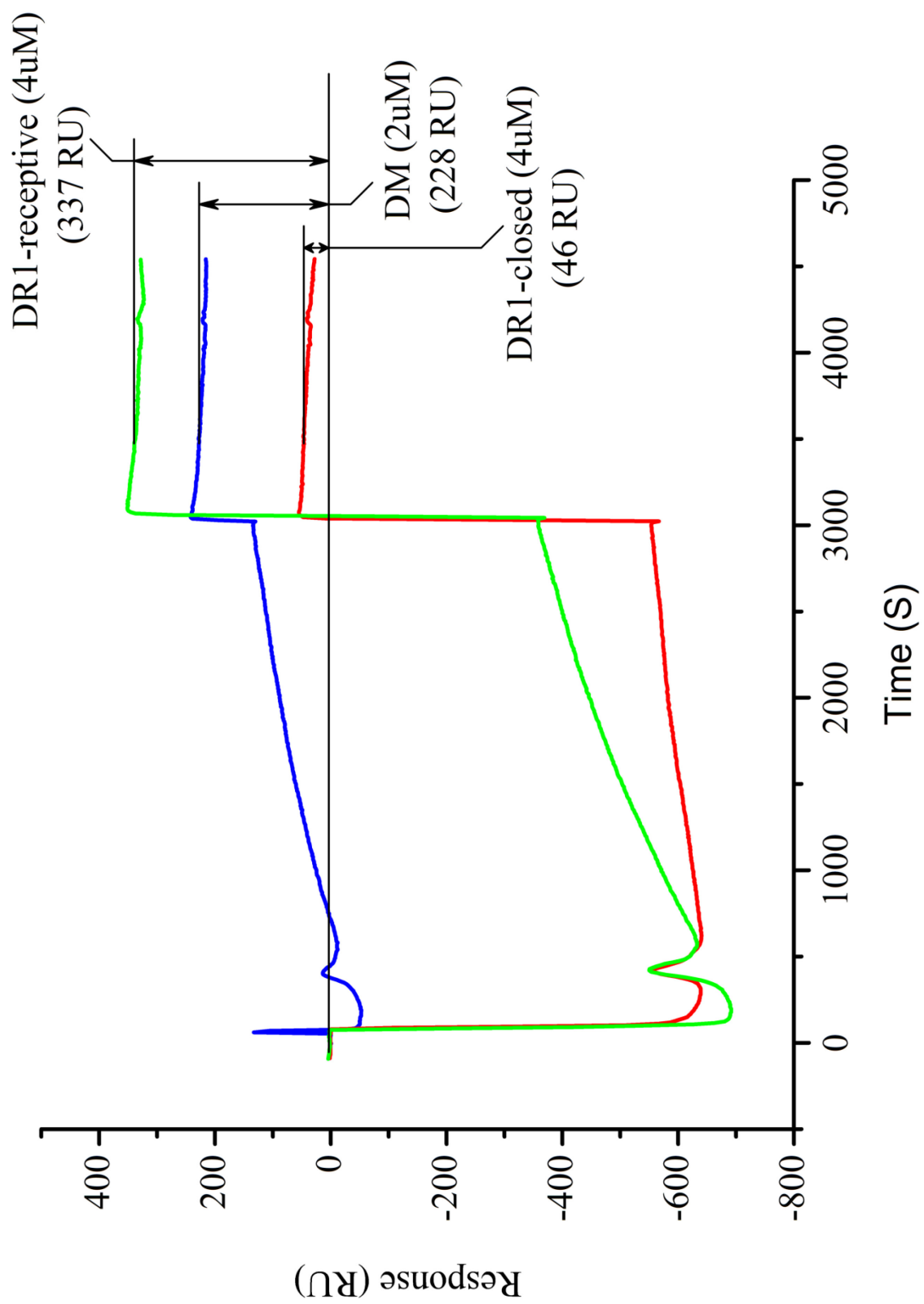
(B) Binding controls of DR1 $\beta$ G86Y and DR1/HA(306-318) with anti-His antibody, DM/DO and DM surfaces. Following the immobilization of anti-His antibody, 4  $\mu$ M DR1 $\beta$ G86Y (red trace) and 4  $\mu$ M DR1/HA(306-318) (black trace) were injected over the immobilized antibody. Upon capturing 2000-3000 RU of DM/DO by the anti-His antibody, 4  $\mu$ M DR1/HA(306-318) was injected over the DM/DO (green trace). In a separate control, 3000 RU of DM was captured by immobilized anti-FLAG antibody. 4  $\mu$ M DR1 $\beta$ G86Y (cyan trace), or 4  $\mu$ M DR1/HA(306-318) (blue trace) was injected over the captured DM.



**A****B**

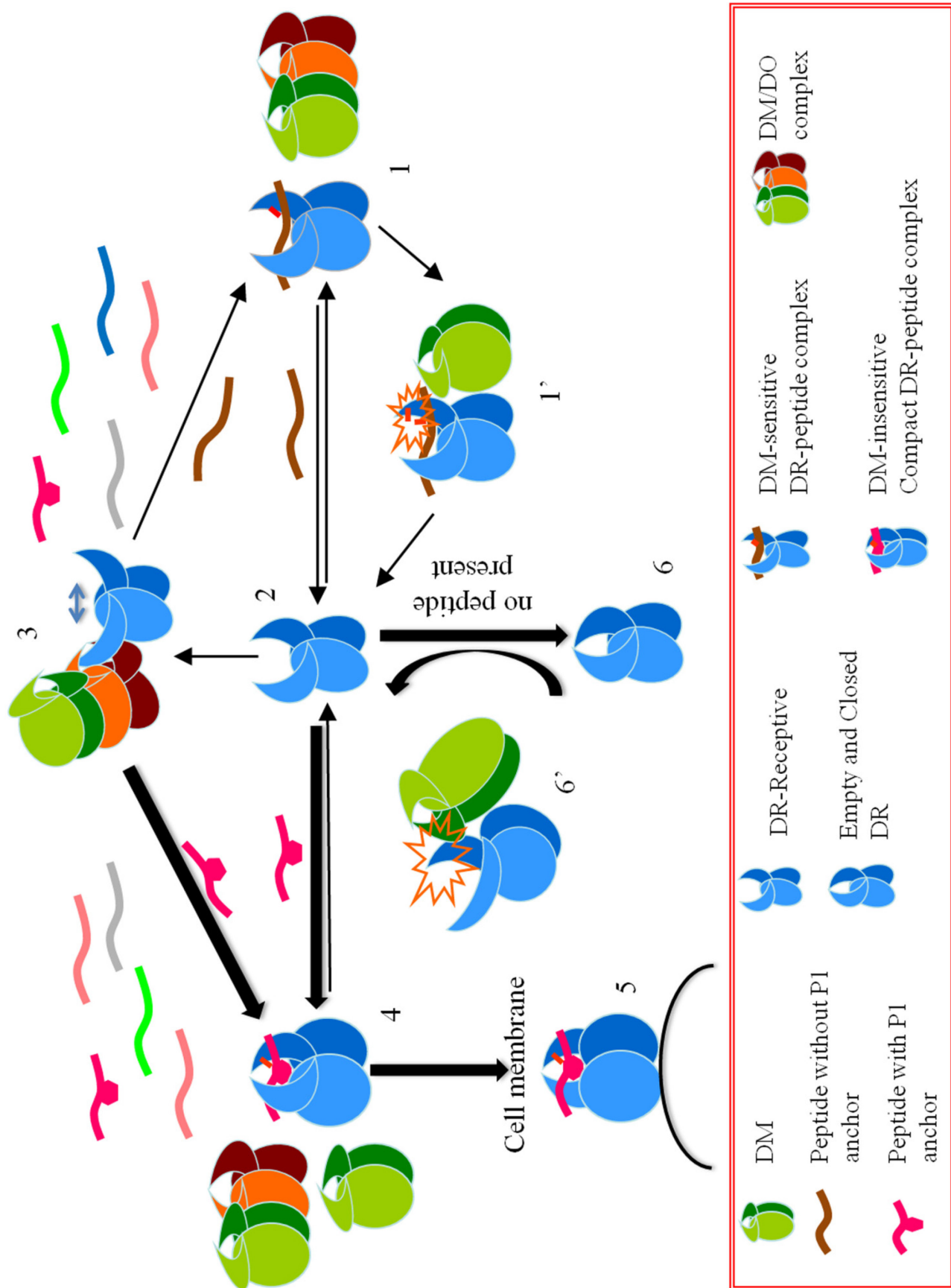
**Figure 7. Transiently receptive DR1 molecules interact with DM/DO complexes.**

Mags.DO5 purified DM/DO was immobilized on anti-His antibody coupled chip surface to a level of 2000-3000 RU. 4  $\mu$ M receptive DR1 with 2  $\mu$ M DM was injected over the captured DM/DO at a rate of 1  $\mu$ l/min and a constant flow-cell temperature of 37°C for 50 minutes (green trace). As a control, prior to the injection of receptive DR1 molecules, 2  $\mu$ M DM (blue trace), or 4  $\mu$ MDR1/HA(306-318) (red trace) were injected at the same flow rate and temperature.



**Figure 8. A model for the mechanism of DO or DM/DO interaction with DR1.**

Starting from a CLIP-bound DM-sensitive conformation (**conformation 1**), DR interacts with DM (**conformation 1'**), and a peptide-receptive open conformation is generated (**conformation 2**). An open conformation can also be induced by DM interacting with empty DR (**conformation 6'**). DO or DM/DO complexes interact with peptide-receptive DR molecules and stabilize an overly receptive conformation (**conformation 3**). In the pool of available peptides those that form DM-sensitive complexes with DR do not get a chance to stabilize in the groove. On the contrary, those peptides that form DM-resistant complexes undergo conformational changes and form DR-compact dimers (**conformation 4**), which are shuttled to the cell membrane (**conformation 5**). If DR-Receptive (conformation 2) does not find a peptide to bind it converts to a closed conformation (**conformation 6**).



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## **Chapter Four**

### **DO can skew the selection of the immunodominant peptide epitope from the antigenic protein**

#### **Introduction**

Our studies on the effect of DO utilized individual peptides and led to a model for its role in class II antigen presentation<sup>1</sup>. However, our model needs to be evaluated against antigenic proteins. When an antigenic protein enters the class II processing system, it is first exposed to the acidic environment with proteases, known as Cathepsins, of the MIIC specialized endosomal compartment. Contrary to most text book illustrations, the digestion of the protein does not occur before its binding to the MHC II molecules. Our lab has observed in numerous occasions that the digestion by Cathepsins is rapid and will result in complete digestion of the antigenic protein with no peptides left to bind the MHC II molecules (Kim et.al, in review). Kim et al demonstrated that immunodominant peptide sequences of the antigen need to be protected by the MHC groove from being digested. Multiple factors contribute to the selection of the immunodominant epitopes that includes; accessibility of the epitopes to MHC II molecules, the binding “affinity” of the exposed epitopes, the specificity of Cathepsins, the activity of the class II accessory molecules, and numerous other factors. It is this very process that determines what peptides will be used to stimulate T cells. In order

to understand the role of DO in our immune system we have to understand how it might affect the selection of immunodominant epitopes from full length proteins.

Our laboratory has recently developed a cell-free antigen processing system for MHC class II <sup>2</sup>. This reductionist system is composed of five recombinant soluble proteins involved in MHC II antigen processing, namely, recombinant DR1, DM and three Cathepsins (Cat), Cat S, Cat B and Cat H. Full-length denatured or native antigenic protein are incubated with DR and DM and then cathepsin are added. DR1, now bound to different peptides is immunoprecipitated and subjected to acid elution of the peptide. Mass Spectrometry is used for the identification of the peptides.

Once the protein binds to DR1, Cathepsins are introduced into the system and any epitope that is not protected by the MHC II groove is digested, leaving behind only the long-lived, generally DM-insensitive, peptides. DR1 is then immunoprecipitated through the L243 Ab resin and the peptides it binds are eluted with trifluoroacetic acid (TFA). Once eluted, the peptides are sequenced and identified via mass spectroscopy (MS).

Using our cell free system, we were able to confirm the sequence of immunodominant peptides determined through other studies<sup>3,4</sup> as well as discover new immunodominant epitopes *de novo*<sup>2</sup>. In our lab, immunodominance is defined by the ability of the peptide to induce the same immunologic recall response in T cells as the full length antigenic protein following the initial immunization. For the most part, we can find the immunodominant epitope of a full length protein by sequencing the peptides eluted in our cell free system. In addition to the immunodominant peptides there are a number of other non-dominant peptides that are eluted from MHC II molecules that do not stimulate T cells from full length protein immunized mice. By utilizing this minimalistic MHC II processing system we are able to examine the effects of DO both with and without DM on the selection of immunodominant epitopes for DR1. Our experiments have produced evidence that the effect of DO is variable for different antigenic proteins. While having little effect on the peptide selection of the H5N1-HA1 protein, DO seems to have a strong effect on the selection of the immunodominant type II collagen (CII) derived peptide.

## **Materials and methods**

### **A. Production of soluble recombinant MHC class II proteins**

See Chapter 2.

## **B. MHC II cell-free processing system and Mass-Spec sample preparation**

Antigenic full length proteins were pre-bound to DR1 with or without accessory molecules in citrate-phosphate pH 5.0 buffers for 3 hours at 37°C. For each experiment 4 samples and a background without any antigenic protein added were prepared:

1: DR1

- protein of interest
- HLA-DR1

2: DR1+DM

- protein of interest
- HLA-DR1
- HLA-DM

3: DR1+DO

- protein of interest
- HLA-DR1
- HLA-DO

4: DR1+DM+DO

- protein of interest
- HLA-DR1
- HLA-DM
- HLA-DO

B: DR1+DM+DO background

- HLA-DR1
- HLA-DM
- HLA-DO

Following the 3 hour incubation, reactions were adjusted for processing by Cathepsins to digest away the protein leaving behind only the DR1-bound epitopes. The solution of DR1/protein with Cathepsins B, H and S (or alternatively V instead of S)(Calbiochem) was incubated for 2 hours at 37°C to allow complete digestion, then the reaction was neutralized by the addition of 0.2M Na<sub>2</sub>HPO<sub>4</sub>. DR1 with bound peptides was immunoprecipitated by L243 conformation specific antibody to wash away the Cathepsins and the accessory molecules. Peptides were eluted from DR1 through the addition of 1% TFA. The eluted peptides were separated from DR1 through the use of a 10 kDa Microcon YM – 10 centrifugal filter unit (Millipore). To prepare the samples for Mass spec, the peptides were dried and reconstituted in 50% methanol with the addition of 100 fmol of Angiotensin I (DRVYIHPFHL) peptide for the generation of a standard peak.

## **Results**

### **A. DO has a minor effect on shifting the repertoire of DR1 molecules when exposed to a mixture of immunodominant peptides**

We set out to determine what the effect of DO and DM accessory molecules is on the selection of immunodominant peptides from a full length antigen. If the effect of accessory molecules on antigen presentation is large enough to affect the onset of disease, then they should significantly alter the sequences of peptides that are presented by MHC II molecules to T cells. Given the ability of DO to regulate the binding of peptides to DR1 and the reports made by some researchers on the effect of DO in model systems, we believe that DO should be able to make changes in T cell repertoire. To support this theory we wanted to find a particular immunodominant epitope the presentation of which would be enhanced, diminished or even completely eliminated by the presence of DO.

Before performing the digestion of a full length antigenic protein, we tested the ability of DO to alter the selection of a peptide by DR1 from a set of known immunodominant and non-dominant peptides. 4 samples of DR1 with or without accessory molecules were incubated in the presence of 250 fmol of HA(306-318), HA(Y308A), CII(259-273), H5N1-HA1(259-274), DR3 restricted Uveitis peptide, HSA(291-306) (NRERRGIALDGKIKHE), and DR3 restricted Thyroglobulin peptide, TG(2098-2112) (LSSVVVDPSIRHFDV) (**Table 1**). After a 3h incubation at 37°C, DR1 molecules with bound peptides were immunoprecipitated by L243 antibody coupled resin. The peptides were eluted from DR1 and

reconstituted in 50% methanol. Angiotensin I (DRVYIHPFHL) was added to each of the 4 samples to generate the standard peak of relative peptide abundance. Samples were analyzed by Matrix-assisted laser desorption/ionization-linear trapping quadrupole (MALDI-LTQ) (Thermo Fisher) mass spectroscopy. MALDI-LTQ is not a quantitative technique and it is hard to know the exact amounts of peptide that each sample contains. To assess a relative estimate of the quantities of peptides for comparison among different samples, we normalized all of our peptide peak signals to the signal generated by the Angiotensin I peptide standard (**Fig 1**). Short-lived peptides which easily dissociate from DR1 and peptides not restricted to DR1 did not show up in the MALDI readings regardless of the presence of DM, or DO. As expected, long-lived peptides insensitive to DM-mediated dissociation outcompeted short-lived ones. While the abundance of CII(259-273) peptide remained relatively constant, the abundance of H5N1-HA1(259-274) and HA(306-318) peptides were increased in the presence of DM, as compared to the sample with no accessory molecules. In support of our previous observations, the samples containing both DM and DO accessory molecules increased the relative abundance of H5N1-HA1(259-274) and HA(306-318) peptides even further as compared to the sample containing DM alone. This is likely the result of a cooperative effect between DO and DM in enhancing the binding of DM-insensitive peptides.



In these experiments DO did not completely prevent the binding of one immunodominant peptide for another in any of the samples. This means that the effect of DO is more subtle than we had hoped for. To observe the effect of DO we would have to perform the selection of peptides from the digestion of a full length protein in our cell free system. This would generate a great number of both long- and short lived peptides for DO to exert its effect on, as well as give us a chance to gauge the subtle changes imposed by DO on the antigenic peptide repertoire.

**B. DO has little effect on the selection of the immunodominant H5N1-HA1(259-274) peptide from the full length H5N1-HA1 protein while still altering the antigenic peptide repertoire**

We were not able to observe the effect of DO on selecting a particular immunodominant peptide that could not be selected by either DR1 alone or DR1 in the presence of DM. To better visualize the effect of DO it was necessary to process a full length antigen both in presence and the absence of DO and to quantitate the relative abundance of both the immunodominant as well as the non-dominant epitopes that remained bound to DR1. This way we would be able to show that DO had a detectible affect on antigen processing and not just the binding of individual peptides.

Our first cell free processing experiment was conducted with a full length influenza H5N1-HA1 protein. We have previously determined the sequence of its immunodominant epitope, which makes it easier to examine if DO has any significant effect on its presentation. Four different samples of DR1 were incubated with H5N1-HA1 protein at 37°C for 3 hours in the presence of DO and DM in combination with each other. Another sample with DR1, DM and DO but no antigenic protein was also incubated at 37°C for 3 hours to serve as a Mass-Spec background control where all peptide peaks not specific for H5N1-HA1 protein would be displayed. Cathepsins B, H and S were then added to each sample to digest away the protein and the unbound peptides. Following a 2 h digestion, DR1 molecules were immunoprecipitated with L243 antibody-coupled resin and the bound peptides were acid eluted from DR1 with TFA. The eluted peptides were run on a MALDI-LTQ mass spectrometer to observe the presence of the immunodominant peaks (**Fig. 2**). Unique peptide peaks in samples were determined by comparing the mass spectra to that of the background sample that did not include the HA protein. A comparison of the abundance of H5N1-HA1(259-274) peptide peaks in samples including DO and DM or DM only suggested that addition of DO did not significantly affect the abundance of HA1(259-274) peptide in the presence of DM. At the same time, unique peaks at 1899 and 2675 Da were detected in samples of DR1 alone and DR1 with DM respectively. This suggests that DO may have diminished the

presentation of a number of non-dominant peptides present in samples without DO.

In addition to sequencing our H5N1-HA1 protein derived peptides eluted from DR1 we performed a relative quantitation of the abundance of each peptide sequence in the sample. For this purpose a "peptide correlation profiling/ion intensities" analysis was performed on each sample. This method takes into account both the time of the peptide elution in the MS sample as well as the intensity of its signal<sup>5,6</sup>. Using this technique we received a list of peptides derived from the H5N1-HA1 protein identified in all 4 samples (**Fig. 3A-D**). Just as we have seen in our MALDI-LTQ profile, no matter what accessory molecules were present, the immunodominant H5N1-HA1(259-274) peptide and its variants with extended N- and C- terminal residues were always detected. However, the amount of the immunodominant peptide in samples with no accessory molecules or just the DO accessory molecule was hundreds of times smaller. This led us to hypothesize that the presentation of the immunodominant H5N1-HA1(259-274) peptide is determined by the presence of DM and DO does not play a role in inhibiting DM. The peptide fragments presented by each set of accessory molecules were derived from the entire sequence of the protein antigens, and few fragments were uniquely presented in samples with combinations of

accessory molecules (**Fig. 3E**). As such, H5N1-HA1(259-274) digested in the presence of DO, or DO and DM displayed the presence of non-dominant epitopes, peptides shown as (59 - DLDGVKPLILR – 69) and (324 - SNRLVLATGLRNSPQ – 338), while other samples did not. While in the case of the H5N1-HA1 protein DO did not significantly affect the presentation of the immunodominant epitope, it was able to alter the total antigenic peptide repertoire.

### **C. DO inhibits the presentation of the immunodominant CII(259-273) peptide fragment processed from a pre-cut type II collagen protein**

The experiment with the processing of the H5N1-HA1 protein showed that the presence of an accessory molecule could significantly alter the relative abundance of immunodominant peptides compared to their non-dominant counterparts. In case of H5N1-HA1, it was DM that had the largest effect on the presentation of the immunodominant peptide. To determine if this remains true for other antigenic proteins we decided to examine the effects of DO on the presentation of the type II collagen (CII) immunodominant epitope. This immunodominant epitope has been previously identified by studies conducted on DR1-transgenic mice<sup>3</sup> and reconfirmed by our cell-free system<sup>2</sup>. Due to a high sequence similarity, including the immunodominant epitope, we used bovine collagen instead

of its human form. Unlike H5N1-HA1, which can be added in its native form to the cell-free system, CII has to be first denatured and digested by MMP9. CII was boiled in acetic acid and pre-digested by MMP8 activated MMP9 before its addition to the cell-free system for processing<sup>2</sup>. After the CII pre-digested fragments bound the DR1 molecules, they were included in cell free processing system and the eluted peptides were analyzed by MALDI-LTQ. It is important to note that CII(259-273) peptide is not as easily digested by Cathepsins as is the H5N1-HA1(259-274) peptide (Kim et.al, in review). The peptide peaks varied from one sample to the next indicating the ability of DO to alter the antigenic peptide repertoire presented by DR1. Unfortunately, we did not detect the immunodominant CII(259-273) epitope in any of the samples (**Fig. 4**). The immunodominant epitope and its different length variants were, however, identified when the samples were analyzed and sequenced (**Fig. 5A-D**). This time the abundance of the CII(259-273) epitope as well as its variants differed in the presence or absence of DO. The immunodominant epitope had the highest abundance in the sample containing DM. The sample containing DO had nearly 25 times less of the epitope, and the sample containing both DM and DO had nearly a 50 fold decrease in the amount of immunodominant epitope presented. Just like with H5N1-HA1, the epitopes presented in the 4 samples of DR1 with and without DM and DO were spread out along the sequence of the protein with some epitopes being unique to only a few combinations of accessory molecules

(**Fig. 5E**). This is our first confirmation of the ability of DO to significantly shift the abundance of the immunodominant peptides selected by DR1 for presentation to T cells.

#### **D. Substitution of cathepsin V for cathepsin S prevents the CII(259-273) epitope from being presented by DR1**

The thymic epithelial cells are some of the few MHC class II presenting cells that have been found to express DO<sup>6,7</sup>. Unlike most MHC II molecules DO has only been found to be expressed in B cells, the thymic epithelial cells and a few subsets of dendritic cells<sup>8</sup>. Specifically, DO is expressed in epithelial cells ringing the Hassall's corpuscles (HC) structures. Not much is known about the true purpose of those structures, although they have been previously implicated in the generation of T regulatory cells<sup>9,10</sup>. We hypothesized that the presence of DO in the thymic epithelia, while being absent from DCs and Macrophages, serves some specific function. To mimic the effect of DO in the thymic epithelial cells we altered the Cathepsins used in our cell free system. In all of our experiments so far, we used Cathepsins B, H and S for the digestion of antigens. Cathepsin S while being important in the MHC II processing system<sup>11,12</sup>, is not expressed in the thymic medullary cells that show expression of DO<sup>13</sup>. Instead thymic epithelial cells express Cathepsin V (also known as L2) or its mouse analog Cathepsin

L<sup>14-16</sup>. As an experiment we decided to substitute Cathepsin S in our system with Cathepsin V and see if in combination with DO the repertoire of peptides would be different.

We repeated our cell-free system experiment with CII protein this time substituting Cathepsin S with Cathepsin V. Surprisingly, the sequencing results showed a complete lack of the immunodominant CII(259-273) epitope or its variants (**Fig. 6A-D**), at the same time, another epitope, not present in the previous experiment was detected in a few samples. The location of the epitopes selected in the presence of Cathepsin V from the sequence of the full length protein was with only a few exceptions very similar to our previous result (**Fig. 6E**). This experiment shows that not only the accessory molecules, but also the proteases in antigen processing can have significant effects on the repertoire of presented peptides.

## **Discussion**

Our cell-free experiments have provided significant insights into the MHC class II antigen presentation system. In our previous studies<sup>2,17</sup>, the DR1 molecules that were used in the cell-free system were preloaded with a short-lived, DM-sensitive peptide – HA(Y308A). DR1 molecules become instantly receptive when HA(Y308A) dissociates, which promotes a better

binding of the antigenic protein during the pre-binding step, before the Cathepsins are introduced. This approach generally resulted in similar levels of immunodominant peptide presented both in the presence and absence of DM. It is important to note that HA(Y308A)<sup>18</sup>, has a similar half-time of dissociation in the presence of DM as does the CLIP peptide<sup>19</sup>. But we do not know how substituting HA(Y308A) for CLIP may affect the final outcome of presented peptide epitopes. In experiments described in Chapter 4 the DR1 used in the cell free system was not pre-loaded with HA(Y308A). We believe that this allowed us to better observe the differences in peptide loading in the presence of accessory molecules. The relative amount of immunodominant H5N1-HA1 as well as CII derived peptides that were eluted from DR1 in the absence of DM and DO is significantly less than the amounts eluted in the presence of DM, DO and even DM and DO together, highlighting the importance of MHC II accessory molecules (**Fig. 3,5**). While we are still not certain how this observation might be translated to the peptide selection process occurring in vivo, our results confirm that the binding of peptides to DR1 depends on DM and DO.

The processing of H5N1-HA1 protein, as observed in our cell-free system, has identified DM as the molecule being primarily responsible for the presentation of its immunodominant epitope. The presence of DM has



increased the relative amount of immunodominant peptide and its various size variants by a factor of 100 as compared to the samples where H5N1-HA1 protein was processed by DR1 alone or DR1 with DO (**Fig. 3**). To our surprise, although DO enhanced the presentation of H5N1-HA1(259-274) peptide (see Chapter 2), it did not increase the relative amount of the immunodominant peptide variants by itself or in the presence of DM. It is important to note that when the same samples were analyzed by spectral counting, we saw an increase in the number of immunodominant H5N1-HA1(259-274) peptide PSMs associated with the sample containing both DM and DO (86 PSMs) as opposed to DM alone (52 PSMs). This necessitates further experimentation to determine the effects of DO on the selection of peptides from a full length antigen. This will allow us to expand on our mechanistic model for the effects of DO, taking into account the data we have already generated for individual peptides.

DO played a primary role in the inhibition of the selection of CII immunodominant epitopes. While the amount of immunodominant epitopes in the presence of DM was not very significant, in samples containing DO and both DM and DO accessory molecules it decreased by more than 20 fold (**Fig. 5**). While we do not know if this decrease is sufficient to prevent rheumatoid arthritis (RA), we have just found our

first evidence of DO having an observable effect in a disease model. This is not the first time DO has been linked to RA. In a population study conducted amongst RA patients a specific SNP in an un-translated region of the HLA-DO $\alpha$  gene was linked to the onset of the disease<sup>20</sup>. We believe that it is important to follow up on this discovery and conduct additional in vivo experiments in DO knock-out mouse models to confirm this association.

Our experiment with an alternate Cathepsin has shown us that not only class II accessory molecules, but the proteases in the MIIC compartment may have a significant effect on the nature of the peptide repertoire presented by MHC II molecules. While we have known about the variable expression of different types of Cathepsins in human tissues<sup>11,13,16</sup>, we have only had associative evidence for their importance in antigen presentation<sup>12,15</sup>. With the establishment of the cell-free system, we can now observe the direct effect that specific Cathepsins may have in the processing of antigens leading to the onset of disease. Our discovery of CII protein being processed differently in the presence of thymic Cathepsin V as compared to Cathepsin S (**Fig. 6**), expressed in DCs, B cells and Macrophages, allows us to speculate on the mechanism of autoimmune disease initiation. While the thymic medullary compartments process CII in addition to many other potential

autoimmune proteins with Cathepsin V, the immunodominant epitopes responsible for the onset of disease are simply not presented. This leads to a lack of negative selection of self-reactive T cells in the thymus. At the same time, CII can be pre-digested and processed in the periphery by antigen presenting cells that express Cathepsin S, which from our experiments is necessary to present the immunodominant epitope. This would allow T cells that escaped negative selection to be triggered by immunodominant CII peptides present in the periphery resulting in autoimmunity. Further experimentation with our cell-free system and additional Cathepsins is required to discern the full extent of this hypothesis. By testing other antigens we may be able to find other cause and effect examples of alternate processing of antigens in different tissues that might lead to the onset of disease.

## Figures

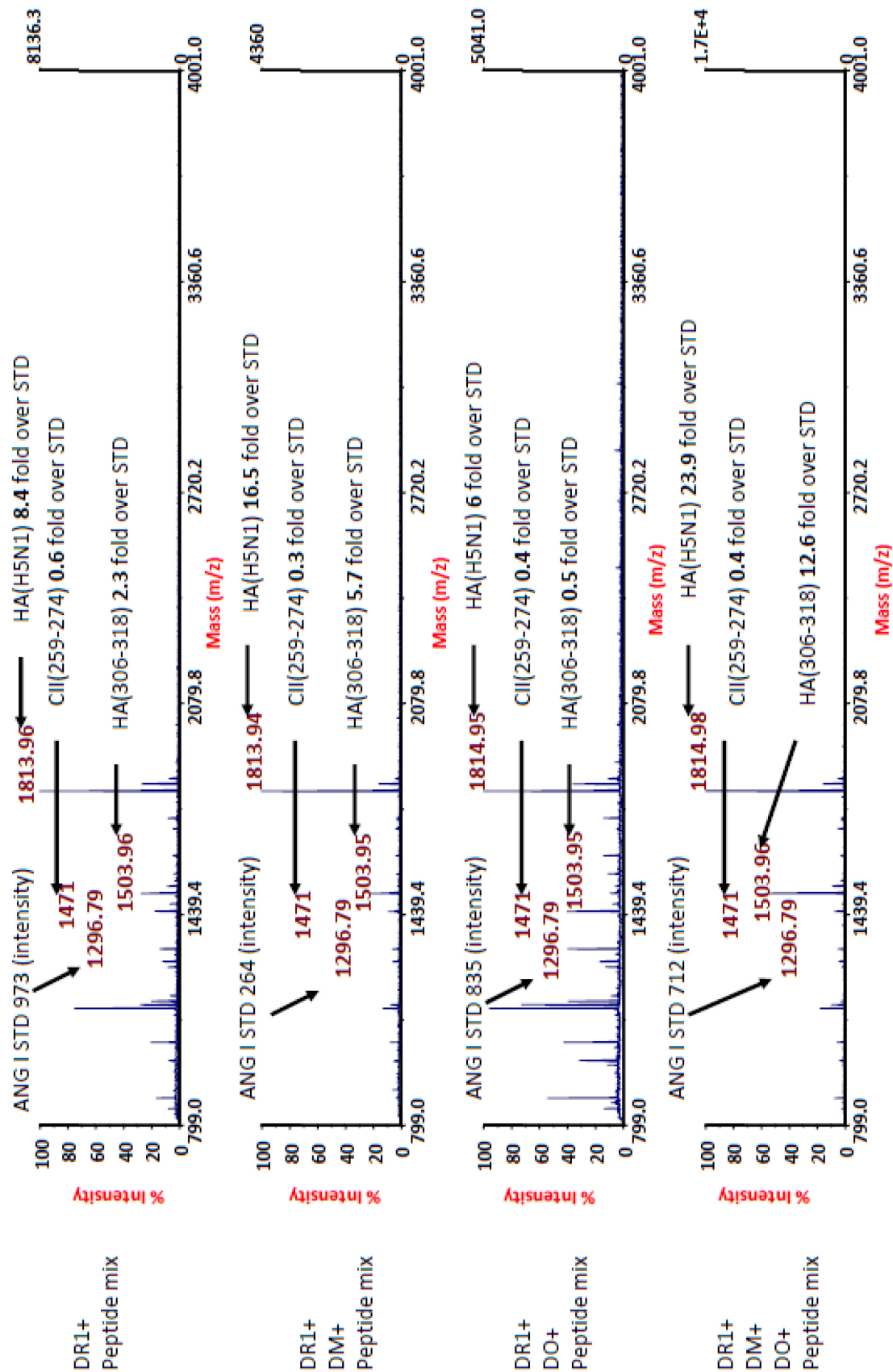
**Table 1. Peptides used to determine the selectivity of DO for peptide binding among immunodominant epitopes.**

List of peptides used in our experiment to test the ability of DO to enhance selection of specific peptides out of a given set. Angiotensin I peptide did not participate in the experiment and was added to the samples later to serve as a standard. Sequence, mass and pI are indicated.

Peptide Name:	Sequence:	pI:	Monoisotopic Neutral Mass (Da):	Amount:
HA(306-318)	PKYVKQNTLKLAT	10	1502.881	250 fmol
HA(Y308A)	PKAVKQNTLKLAT	10.3	1410.855	250 fmol
CII(259-273)	AGFKGEQGPKGEPGP	6.25	1470.75	250 fmol
H5N1-HA1(259-274)	SNGNFIAPEYAYKIVK	8.2	1813.94	250 fmol
(Uveitis peptide)HSA(291-306)	NRERRGIALDGKIKHE	10	1892.049	250 fmol
(Thyroglobulin peptide)(TG(2098-2112)	LSSVVVDPSIRHFDV	5.69	1668.882	250 fmol
Angiotensin I (Added after processing)	DRVYIHPFHL	6.9	1295.677	250 fmol

**Figure 1. MALDI-LTQ spectrum of peptides eluted from DR1 following the incubation with a set of immunodominant peptides.**

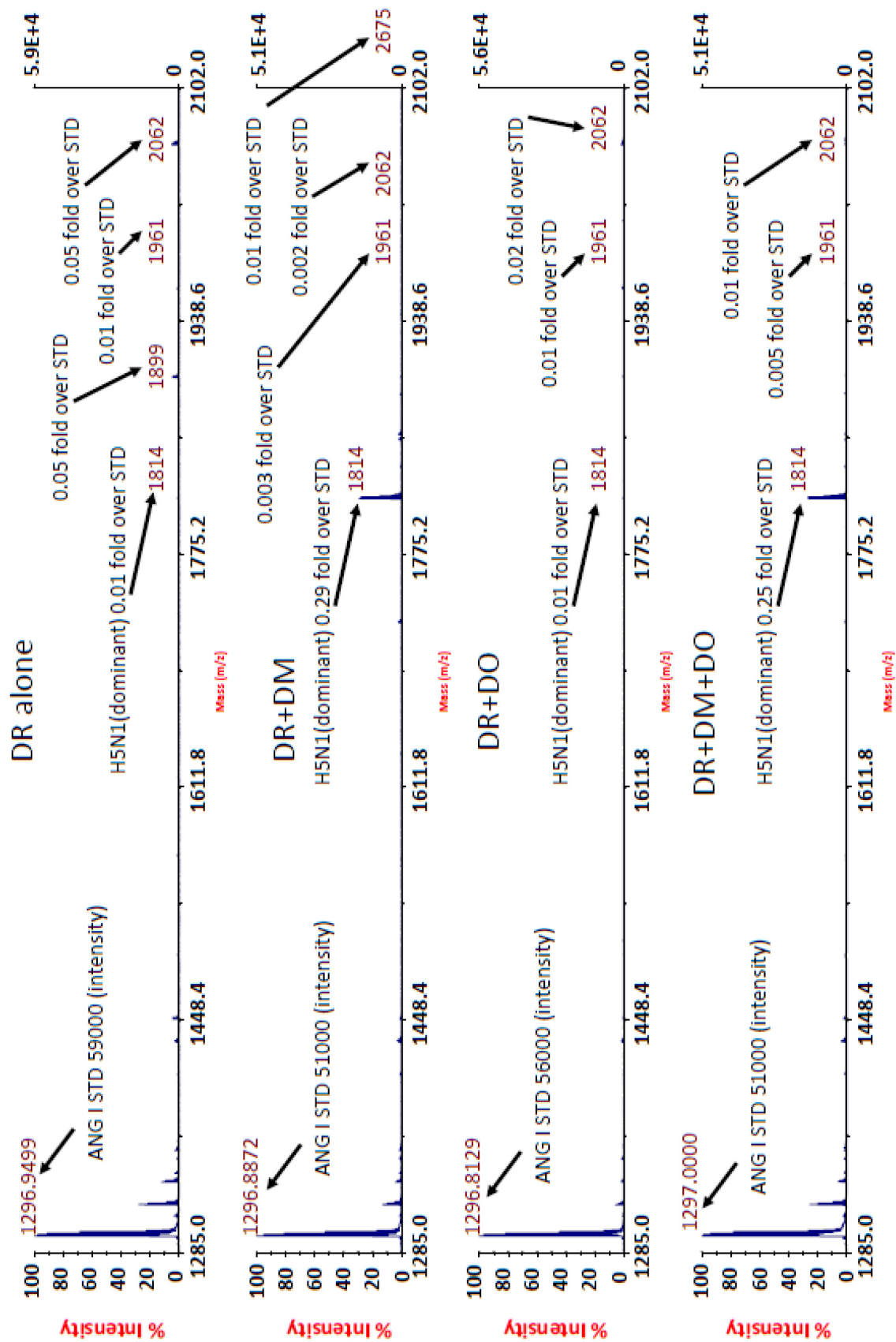
Peptides listed in Table 1, with exception of Angiotensin I, were incubated with DR1 for 3 hours at 37°C with either DR1 alone, DR1 and DM, DR1 and DO, DR1 and DM and DO. DR1 with bound peptides was purified through immunoprecipitation by L243 Antibody resin. Peptides were eluted by exposing DR1 to 1% TFA. Eluted peptides were separated from empty DR1 by filtration through a 10 kDa size exclusion spin filter, dried and reconstituted in 50% methanol with 250 fmol of Angiotensin I peptide. The peptide samples were analyzed by MALDI-LTQ mass spectroscopy at the mass range displaying the peaks of all the peptides in the mix.



**Figure 2. MALDI-LTQ spectrum of peptides eluted from DR1 following the processing of H5N1-HA1 protein.**

Full length H5N1-HA1 protein was incubated with DR1 for 3 hours at 37°C with either DR1 alone, DR1 and DM, DR1 and DO, DR1 and DM and DO. A control incubation of DR1 with DM and DO and no protein was also performed for a sample to serve as a background control for the experiment. DR1 with bound peptides was purified through immunoprecipitation by L243 Antibody resin. Peptides were eluted by exposing DR1 to 1% TFA. Eluted peptides were separated from empty DR1 by filtration through a 10 kDa size exclusion spin filter, dried and reconstituted in 50% methanol with 250 fmol of Angiotensin I peptide. Each identified peak was compared to the spectrum of the background sample. If an identified peak was present in the background it was excluded from the figure, since it was not generated by the H5N1-HA1 protein digestion.





**Figure 3. Mass Spec sequencing of peptides eluted from DR1 following the processing of H5N1-HA1 protein and relative quantitation.**

Full length H5N1-HA1 protein was processed in our cell-free system by Cathepsins – B,H and S in 4 different samples containing DR1 and combinations of DM and DO: DR1 alone, DR1 + DM, DR1 + DO, DR1 + DM + DO. A control sample was processed without the addition of any H5N1-HA1 protein but with DR1, DM and DO, to serve as background control. Peptides present in each sample were sequenced by Mass spectrometry and the relative abundance of each was quantitated by peptide correlation profiling/ion intensities in Dr. Leonard Foster's lab.

(A) Peptides sequenced from the DR1 alone sample and their relative quantities.

(B) Peptides sequenced from the DR1 + DM sample and their relative quantities.

(C) Peptides sequenced from the DR1 + DO sample and their relative quantities.

(D) Peptides sequenced from the DR1 + DM + DO sample and their relative quantities.

(E) Sequence of the full length H5N1-HA1 protein and the location of the identified peptides within it. Each core sequence of peptides was color coded: Yellow – identified in DR1 alone sample; Green – identified in DR1 + DM sample; Turquoise – identified in DR1 + DO sample; Red – identified in DR1 + DM + DO sample.

**A** DR1 alone + H5N1-HA1 + CatB + CatH + CatS:

Peptides:	Relative abundance:
259 - SNGNFIAPEYAYKIVK - 274 (immunodominant)	3.36
264 - IAPEYAYKIVK - 274 (immunodominant)	0.72
208 - QNPPTYISVGTSTLNQRLVPR - 228	1.46
247 - TILKPNDAINFE - 258	0.93
107 - YPGDFNDYEELKH - 119	0.47

**B** DR1+ DM + H5N1-HA1 + CatB + CatH + CatS:

Peptides:	Relative abundance:
259 - SNGNFIAPEYAYKIVK - 274 (immunodominant)	490.61
259 - SNGNFIAPEYAYKI - 272 (immunodominant)	4.25
258 - ESNGNFIAPEYAYKIVK - 274 (immunodominant)	11.22
259 - SNGNFIAPEYAYKIVKKGDS - 278 (immunodominant)	0.96
260 - NGNFIAPEYAYKIVK - 274 (immunodominant)	2.33
261 - GNFIAPAYAYKIVK - 274 (immunodominant)	1.45
262 - NFIAPEYAYKIVK - 274 (immunodominant)	0.61
84 - DEFINVPEWSYIVEKANPVN - 103	1.19
89 - VPEWSYIVEKANPVN - 103	1.92
208 - QNPPTYISVGTSTLNQRLVPR - 228	15.30
209 - NPPTYISVGTSTLNQRLVPR - 228	1.53
244 - FFWTILKPNDAIN - 256	4.42
244 - FFWTILKPNDAINFE - 258	8.54
245 - FWTILKPNDAIN - 256	7.62
245 - FWTILKPNDAINFE - 258	7.91
246 - WTILKPNDAIN - 256	2.78
246 - WTILKPNDAINFE - 258	15.97
247 - TILKPNDAINFE - 258	0.63

**C** DR1+ DO + H5N1-HA1 + CatB + CatH + CatS:


Peptides:	Relative abundance:
259 - SNGNFIAPEYAYKI - 272 (immunodominant)	3.12
259 - SNGNFIAPEYAYK - 271 (immunodominant)	0.59
324 - SNRLVLATGLRNSPQ - 338	2.72
59 - DLDGVKPLILR - 69	0.60
84 - DEFINVPEWSYIVEK - 98	0.88
208 - QNPPTYISVGTSTLNQRLVPR - 228	11.80
209 - NPPTYISVGTSTLNQRLVPR - 228	1.58
246 - WTILKPNDAINFE - 258	0.54
247 - TILKPNDAINFE - 258	4.81
247 - TILKPNDAINFESNGNF - 263	0.74

**D** DR1+ DO + DM + H5N1-HA1 + CatB + CatH + CatS:

Peptides:	Relative abundance:
259 - SNGNFIAPEYAYKIVK - 274 (immunodominant)	312.31
259 - SNGNFIAPEYAYKI - 272 (immunodominant)	2.63
258 - ESNGNFIAPEYAYKIVK - 274 (immunodominant)	6.46
260 - NGNFIAPEYAYKIVK - 274 (immunodominant)	1.96
324 - SNRLVLATGLRNSPQ - 338	0.83
84 - DEFINVPEWSYIVEKANPVN - 103	2.24
208 - QNPTTYISVGTSTLNQRLVPR - 228	19.95
209 - NPTTYISVGTSTLNQRLVPR - 228	3.60
244 - FFWTILKPNDAIN - 256	1.07
244 - FFWTILKPNDAINFE - 258	3.04
245 - FWTILKPNDAIN - 256	2.96
245 - FWTILKPNDAINFE - 258	2.82
246 - WTILKPNDAIN - 256	3.98
246 - WTILKPNDAINFE - 258	9.93
247 - TILKPNDAINFE - 258	3.86


**E**

MEKIVLLFAIVSLVKSQICIGYHANNSTEQVDTIMEKNVTVTTHAQDILEKKHNGKLCD  
 LDGVKPLILRDCSVAGWLLGNPMCDEFINVPEWSYIVEKANPVNDLCYPGDFNDYEELK  
 HLLSRINHFEKIQIIPKSSWSSHEASLGVSACPYQKSSFFRNVVWLIKKNSTYPTIK  
 RSYNNTNQEDLLVLWGIHHPNDAAEQTKLYQNPTTYISVGTSTLNQRLVPRIATRISKVN  
 GQSGRMEFFWTILKPNDAINFESNGNFIAPEYAYKIVKGDSTIMKSELEYGNCNTKCQ  
 TPMGAINSSMPFHNHPLTIGECPKYVKSRLVLATGLRNSPQRERRRKKHHHHHH

DR1 alone -  Underlined - immunodominant epitope

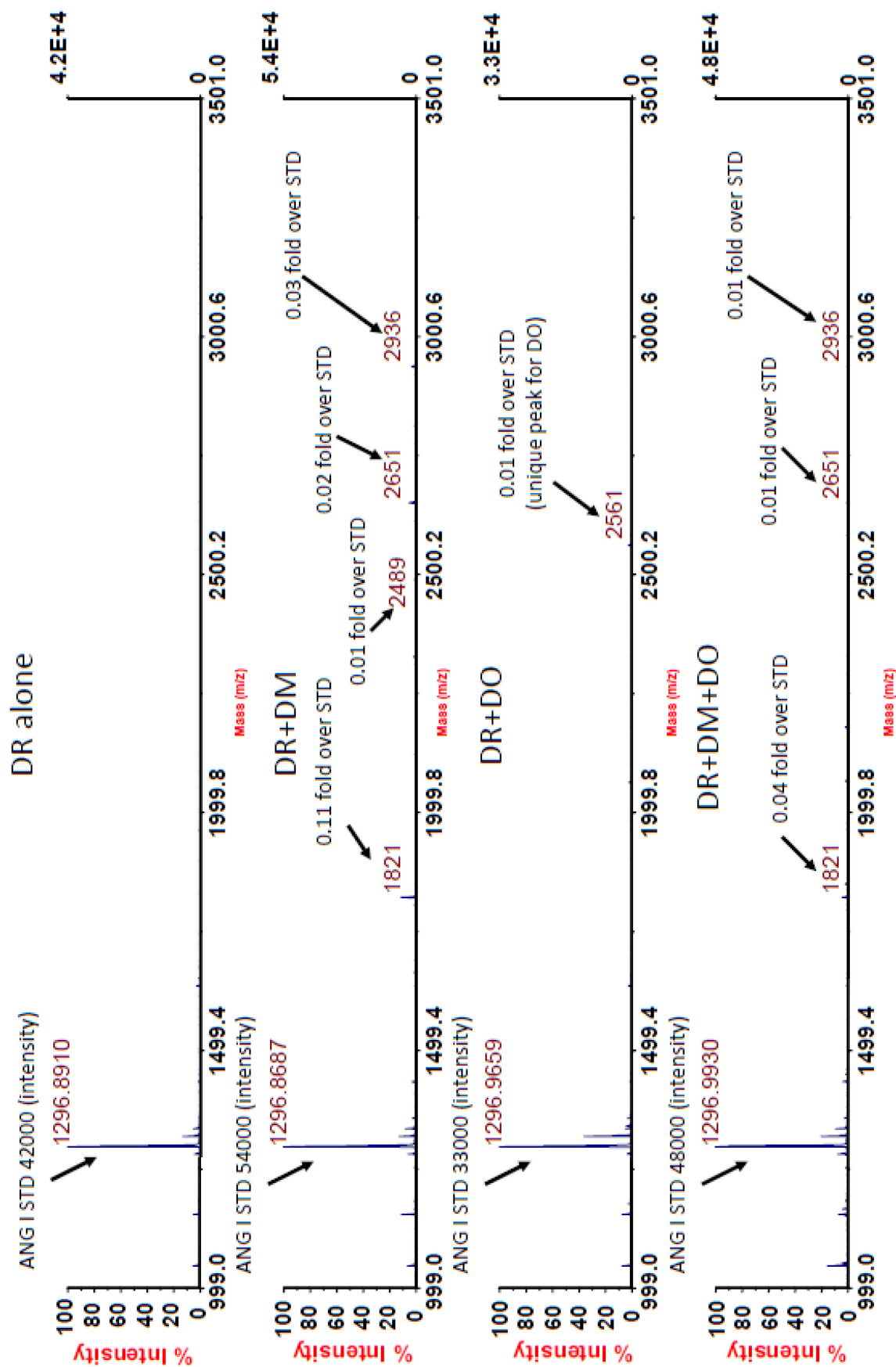
DR1 + DM - 

DR1 + DO - 

DR1 + DM + DO - 

**Figure 4. MALDI-LTQ spectrum of peptides eluted from DR1 following the processing of CII protein.**

Full length CII protein was incubated with DR1 for 3 hours at 37°C with either DR1 alone, DR1 and DM, DR1 and DO, DR1 and DM and DO. A control incubation of DR1 with DM and DO and no protein was also performed for a sample to serve as a background control for the experiment. DR1 with bound peptides was purified through immunoprecipitation by L243 Antibody resin. Peptides were eluted by exposing DR1 to 1% TFA. Eluted peptides were separated from empty DR1 by filtration through a 10 kDa size exclusion spin filter, dried and reconstituted in 50% methanol with 250 fmol of Angiotensin I peptide. Each identified peak was compared to the spectrum of the background sample. If an identified peak was present in the background it was excluded from the figure, since it was not generated by the CII protein digestion.



**Figure 5. Mass Spec sequencing of peptides eluted from DR1 following the processing of CII protein and relative quantitation.**

Full length CII protein was processed in our cell-free system by Cathepsins – B,H and S in 4 different samples containing DR1 and combinations of DM and DO: DR1 alone, DR1 + DM, DR1 + DO, DR1 + DM + DO. A control sample was processed without the addition of any CII protein but with DR1, DM and DO, to serve as background control. Peptides present in each sample were sequenced by Mass spectrometry and the relative abundance of each was quantitated by peptide correlation profiling/ion intensities in Dr. Leonard Foster's lab.

(A) Peptides sequenced from the DR1 alone sample and their relative quantities.

(B) Peptides sequenced from the DR1 + DM sample and their relative quantities.

(C) Peptides sequenced from the DR1 + DO sample and their relative quantities.

(D) Peptides sequenced from the DR1 + DM + DO sample and their relative quantities.



(E) Sequence of the full length CII protein and the location of the identified peptides within it. Each core sequence of peptides was color coded: Yellow – identified in DR1 alone sample; Green – identified in DR1 + DM sample; Turquoise – identified in DR1 + DO sample; Red – identified in DR1 + DM + DO sample.

**A** DR1 alone + bCII + CatB + CatH + CatS:

Peptides:	Relative abundance:
321 - GPRGEPGTPGSPGPAGAAGN - 340	0.71
679 - LQGMPGERGAAG - 690	8.82
1066 - FTGLQGLPGPPGPSG - 1080	0.28

**B** DR1 + DM + bCII + CatB + CatH + CatS:

Peptides:	Relative abundance:
388 - EPGIAGFKGEQGPKGEPGPAGPQG - 411 (immunodominant)	4.64
391 - IAGFKGEQGPKGEPGPAGPQG - 411 (immunodominant)	6.54
396 - GEQGPKGEPGPAGPQG - 411 (immunodominant)	0.20
1062 - GHRGFTGLQGLPGPP - 1076	2.24
1062 - GHRGFTGLQGLPGPPGPSG - 1080	5.27
1066 - FTGLQGLPGPPGPSG - 1080	1.92

**C** DR1 + DO + bCII + CatB + CatH + CatS:

Peptides:	Relative abundance:
388 - EPGIAGFKGEQGPKGEPGPAGPQG - 411 (immunodominant)	0.20
321 - GPRGEPGTPGSPGPAGAAGN - 340	0.12
595 - FQGLPGPPGPPG - 606	0.04


**D** DR1 + DM + DO + bCII + CatB + CatH + CatS:

Peptides:	Relative abundance:
391 - IAGFKGEQGPKGEPGPAGPQG - 411 (immunodominant)	0.15
396 - GEQGPKGEPGPAGPQG - 411 (immunodominant)	0.08
595 - FQGLPGPPGPPG - 606	0.08
679 - LQGMPGERGAAG - 690	0.47
1062 - GHRGFTGLQGLPGPP - 1076	1.10
1062 - GHRGFTGLQGLPGPPGPSG - 1080	2.11

# E


bCII + CatB + CatH + CatS:

Q MAGGFDEKAGGAQLGVMQGPMPGMPRGPPGPAGAPGPQGFQGNPGEPEGVSGPMG  
 PRGPPGPPGKPGDDGEAGKPKSGERGPPGPQGARGFPGTPLPGVKGHRGYPGLDGAK  
 GEAGAPGVKGESGSPGENSGPMPGPRGLPGERGRTGPAGAAGARGNDGQPGPAGPPGP  
 VGPAGGPGFPGAPGAKGEAGPTGARGPEGAQ**QPRGEPGTPGS**PGPAGAAGNPSTDGIPG  
 AKGSAGAPGIAGAPGFPGPRGPPGPQGATGPLGPKGQTGEPIAGFK**GEQGPKEGPA**  
**GPQG**APGPAGEEGKRGARGEPPGAGPAGPPGERGAPGSRGFPGQDGIAGPKGPPGERGP  
 SGLAGPKGANGDPGRPGEPLPGARGLTGRPGDAGPQKVGPSGAPGEDGRPGPPGPQG  
 ARGQPGVMGFPGPKGANGEPPKAGEKGLPGAPGLRGLPGKDGETGAAGPPGPAGPAGER  
 GEQGAPGPSG**FOGLPGFP**GGEGGKPGDQGVPEAGAPGLVGPRGERGFPGERGSFGA  
 QGLQGARGLPGTPTDGPKGASGPAGPPGAQGP**GLQMPGERGAAG**IAGPKGDRGDVG  
 EKGPEGAPGKDGGRGLTGPIGPPGPAGANGEKGEVPPGPAGTAGARGAPGERGETGPP  
 GPAGFAGPPGADGQPGAKEQGEAGQKGDAGAPGPQGPSGAPGPQGPTGVTGPKGARGA  
 QGPPGATGFPGAAGRVPGPSNGNPGP**PPGPS**KDGPKGARGDSGPPGRAGDPGLQG  
 PAGPPGEKGEFGDDGPSGPDGPPGPQGLAGQRGIVGLPGQRGERGFPLPGPSGEPGKQ  
 GAPGASGDRGPPGPVGPPLTGTPAGEPGREGSPGADGPPGRDGAAGVKGDRGETGAVGA  
 PGAPGPPGPSPGPAGPTGKQGDRGEAGAQQGMPGSPGAGARGIQGPQGPRGDKGEAGEPG  
 ERGLKGHRG**FTGLQGLPGFP**SGDQASGPAGPSGPRGPPGPVGPSPGKDANGIPGPI  
 GPPGPRGRSGETGPAGPPGNPGPPGPPGPPGPGID

DR1 alone -  Underlined - Immunodominant epitope

DR1 + DM - 

DR1 + DO - 

DR1 + DM + DO - 

**Figure 6. Mass Spec sequencing of peptides eluted from DR1 following the processing of CII protein in the presence of Cathepsin V and relative quantitation.**

Full length CII protein was processed in our cell-free system by Cathepsins – B,H and V in 4 different samples containing DR1 and combinations of DM and DO: DR1 alone, DR1 + DM, DR1 + DO, DR1 + DM + DO. A control sample was processed without the addition of any CII protein but with DR1, DM and DO, to serve as background control. Peptides present in each sample were sequenced by Mass spectrometry and the relative abundance of each was quantitated by peptide correlation profiling/ion intensities in Dr. Leonard Foster's lab.

(A) Peptides sequenced from the DR1 alone sample and their relative quantities.

(B) Peptides sequenced from the DR1 + DM sample and their relative quantities.

(C) Peptides sequenced from the DR1 + DO sample and their relative quantities.

(D) Peptides sequenced from the DR1 + DM + DO sample and their relative quantities.

(E) Sequence of the full length CII protein and the location of the identified peptides within it. Each core sequence of peptides was color coded: Yellow – identified in DR1 alone sample; Green – identified in DR1 + DM sample; Turquoise – identified in DR1 + DO sample; Red – identified in DR1 + DM + DO sample.

**A**     DR1 alone + bCII + CatB + CatH + CatV:

Peptides:	Relative abundance:
679 - LQMPGERGAAG - 690	5.44
1066 - FTGLQGLPGPPGPSG - 1080	1.55

**B**     DR1 alone + DM + bCII + CatB + CatH + CatV:

Peptides:	Relative abundance:
679 - LQMPGERGAAG - 690	3.08
913 - IVGLPGQRGERG - 924	7.02
1066 - FTGLQGLPGPPGPSG - 1080	75.37

**C**     DR1 alone + DO + bCII + CatB + CatH + CatV:

Peptides:	Relative abundance:
679 - LQMPGERGAAG - 690	3.83
913 - IVGLPGQRGERG - 924	0.63
1066 - FTGLQGLPGPPGPSG - 1080	59.94

**D**     DR1 alone + DM + DO + bCII + CatB + CatH + CatV:

Peptides:	Relative abundance:
595 - FQGLPGPPGPPG - 606	0.07
679 - LQMPGERGAAG - 690	0.99
1066 - FTGLQGLPGPPGPSG - 1080	51.20

## E

bCII + CatB + CatH + CatV:

QMAGGFDEKAGGAQLGVMQGPMPGMPRGPFPAGAPGPQGFQGNPGEPEGPVSGPMG  
PRGPPGPPGKPGDDGEAGKPGKSGERGPPGPQGARGFPGTPLPGVKHGRGYPGLDGAK  
GEAGAPGVKGESGSPGENSGPMPGPRGLPGERGRTGPAGAAGARGNDGQPGPAGPPGP  
VGPAGGPGFPAGPAKGEAGPTGARGPEGAQGPGEPTPGSPGPAGAAGNPSTDGIPG  
AKGSAGAPGIAGAPGFPGRGPPGPQATGPLGPKGQTGEPIAGFKGEQGPKEPGEPA  
GPQAGPAPAGEEGKRGARGEPPGAGPAGPPGERGAPGSRGFPQDGIAGPKGPPGERGP  
SGLAGPKGANGDPGRPGEPLPGARGLTGRPGDAGPQGVGSPGAPGEDGRPPGPPQGP  
ARGQPGVMGFPGPKEGANGEPPKAGEKGLPGAPGLRGLPGKDGETGAAGPPGPAGPAGER  
GEQAPGPSG**FQGLPGPPGPPG**EGGKPGDQGVPEAGAPGLVGPRGERGFPGERGSFGA  
QGLQGARGLPGTPTDGPKGASGPAGPPGAQGPGL**LOGMPGERGAAG**IAGPKGDRGDVG  
EKGPEGAPGKDGGRGLTGPIGPPGPAGANGEKGEVGPVPAGTAGARGAPGERGETGPP  
GPAGFAGPPGADGQPGAKEQGEAGQKGDAGAPGPQGPSGAPGPQGPPTGVTGPKGARGA  
QGPPGATGFPGAAGRVGPPGSNGNPFPFPGPSKDGPKGARGDSGPPGRAGDPGLQG  
PAGPPGEKEGEPGDDGPSGPDGPPGPQGLAGQRG**IVGLPGQRG**ERGFPGLPGPSGEPPKQ  
GAPGASGDRGPPGPVGPGLTGPAEPGREGSPGADGPPGRDGAAGVKGDRGETGAVGA  
PGAPGPPGSPGPAGPTGKQDRGEAGAQPMPGPSGPAGARGIQGPQGPGRDKGEAGEPG  
ERGLKGHRG**FTGLQGLPGPPGPS**GDQASGPAGPSGPRGPPGPVGPSPKDGANGIPGPI  
GPPGPRGRSGETGPAGPPGNPFPFPFPFPFPFP

DR1 alone -  Underlined - Immunodominant epitope

DR1 + DM - 

DR1 + DO - 

DR1 + DM + DO - 

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## **Chapter Five**

### **Conclusions**

Our experiments have generated a great number of critical observations concerning the effects of the DO molecule on antigen presentation by MHC class II DR1. This allows us to present a unifying hypothesis for the role of DM and DO in antigen presentation.

In the scope of our biochemical experiments we have observed that DO can have both enhancing and diminishing effects on the binding of peptides. Observations of this sort have been reported before with little or no follow-up. At the same time, our studies were able to establish a reliable link between the half-time of a peptide's dissociation, its sensitivity to the effects of DM and the effect DO would have on it. As noted in Chapter 2, both the quality and the magnitude of the effect of DO was related to the half time of dissociation of the protein, much as it is in the case of the well studied DM accessory molecule. Unlike previously suggested, we have shown that the mechanism of DO is not related to that of DM and that DO is able to interact with DR1 on its own. This became evident when we demonstrated that DO can have both enhancing and diminishing effects on DR1 molecules pre-loaded with a short-lived peptide. The separate nature of the mechanisms of DO and DM is something that manifests itself in most of our experiments

described in Chapters 2 and 3. While a significant finding on its own, we were able to expand our studies and determine the exact mode of interaction between DO and DR1. By experimenting with a mutant DR1 molecule that was stabilized in a receptive conformation we found that DO could still affect its binding of peptides. Since the mutant DR1 molecule could not adopt the closed conformation, this means that the mechanism of the effect of DO does not involve forcing the DR1 molecules to become receptive. At the same time, dissociation experiments in Chapter 2 have shown that DO has little to no effect on the dissociation of peptides. We hypothesized that this was because DO could not interact with a closed or compact conformation of the DR1 molecule. Our hypothesis was proven correct with our SPR binding experiments depicted in Chapter 3 which showed that DO can only interact with receptive DR1 molecules but not the closed compact conformers.

To explain our observations we propose a unifying theory of the mechanism of DM and DO in Class II antigen presentation. We theorize that DO requires DM to generate the peptide-receptive conformation in DR1 molecules, while at the same time imposing its level of selection on the antigenic peptides processed by the Class II system. When the DR1 molecules become receptive, DO is able to exert its effect and enhance the presentation of some peptides (most likely long-lived) while

diminishing the presentation of other peptides (most likely short-lived). This way DO would impose another level of selection on the antigenic peptide processing in addition to that of DM. The presence of DO most likely results in a narrower peptide repertoire than that generated by DM alone.

As a validation of our theory behind the effect of DO and DM, our full length antigen processing experiments described in Chapter 4 have shown that the nature of the peptides bound to DR1 is different in the presence of DO, DM and DO with DM together. By utilizing our cell-free processing system we have discovered that certain antigens present their immunodominant peptides better in the presence of DM with DO having little or no effect on the amount of peptide presented. At the same time we found that the presence of DO could significantly alter the presentation of the immunodominant peptide derived from type II collagen processing, to the point where it is barely detectable. This opens up a whole new research approach in studying Class II antigen presentation. By thoroughly experimenting with the processing of antigenic proteins we will be able to find more examples of accessory molecules playing a significant role in antigen presentation and perhaps even disease pathology. This, in turn, opens up new possibilities to apply treatment through affecting the MHC class II accessory molecules through chemical and biological means. In addition, we have provided

evidence for how the variable processing of antigens in different immunological compartments that contain different class II digestive enzymes may lead to autoimmunity. While more research has to be done to follow up on our discoveries, it is evident that experiments with our cell-free system have to be expanded to accommodate the fact that DO as well as particular Cathepsins are expressed only in specific cells of the immune system.

## **Appendix i**

### **Discovering the altered receptive conformation in DR1 molecules through circular dichroism**

DO binds to a receptive conformation of DR1. We theorized that upon binding DO would force a receptive DR1 molecule to adopt a theoretical “super-receptive” conformation. This conformation would have a peptide groove more open than that in a peptide-receptive DR1, which would make the DR1 molecule less stable. If this is the case then the thermal stability of DR1 would be diminished even further in the presence of DO. To test this we measured the circular dichroism (CD) of DR1 alone, DO alone and DR1 together with DO. The concentrations had to be adjusted to make sure that the signal generated by DO would not overtake the signal generated by DR1 (**Fig. 1**).

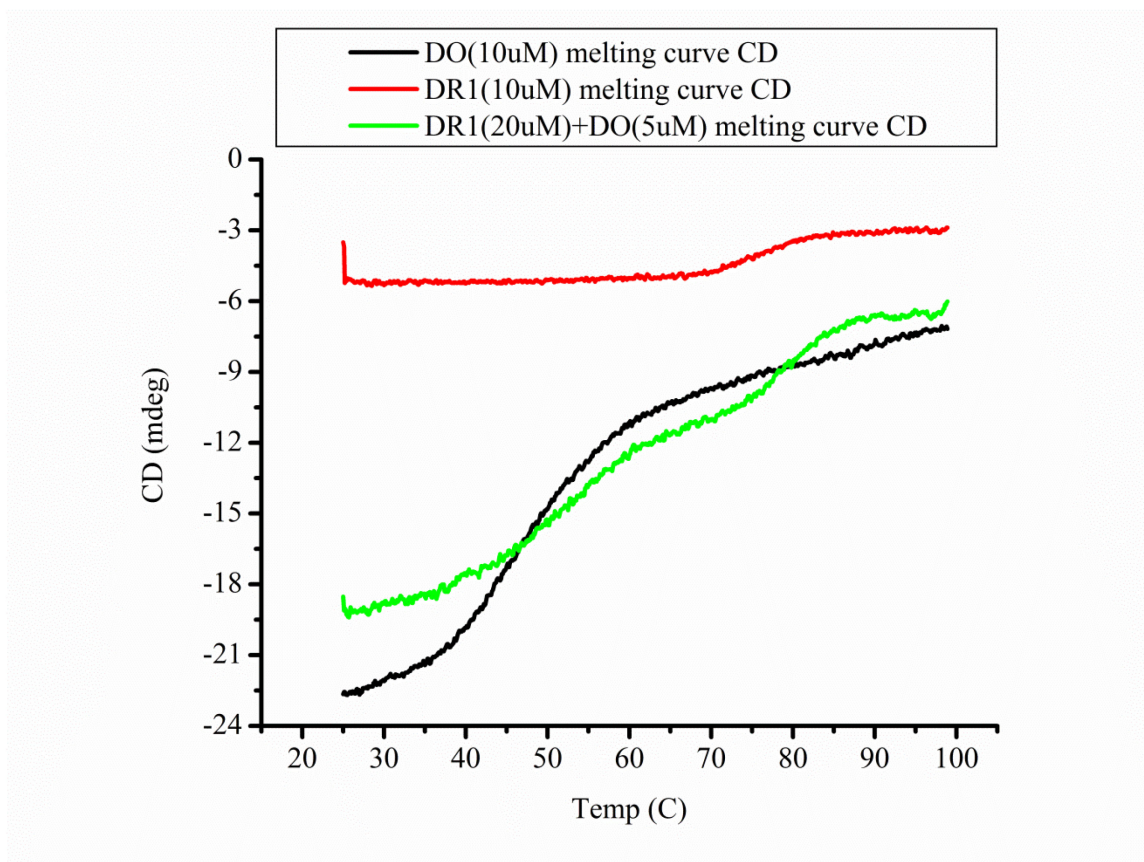
Additionally the same experiment was repeated with a receptive DR1 molecule. DR1 was preloaded with Y308A peptide for 3 days and excess peptide was removed before the start of the experiment, while the samples were kept on ice. The CD of DR1-HA(Y308A) alone, DO alone and DR1-HA(Y308A) together with 5  $\mu$ M DO was determined using the same parameters as before (**Fig. 2**). In each case the temperature changes of the transition points were minor and DO seemed to have made DR1 more stable.

**Figure 1. Circular dichroism melting curve scan of DR1 in the presence of DO**

(A) CD scan of 10  $\mu\text{M}$  DR1 alone, 10  $\mu\text{M}$  DO alone and 20  $\mu\text{M}$  DR1 together with 5  $\mu\text{M}$  DO over a temperature range of 25  $^{\circ}\text{C}$  to 100  $^{\circ}\text{C}$ , with an increase rate of 1  $^{\circ}\text{C}/\text{min}$ .

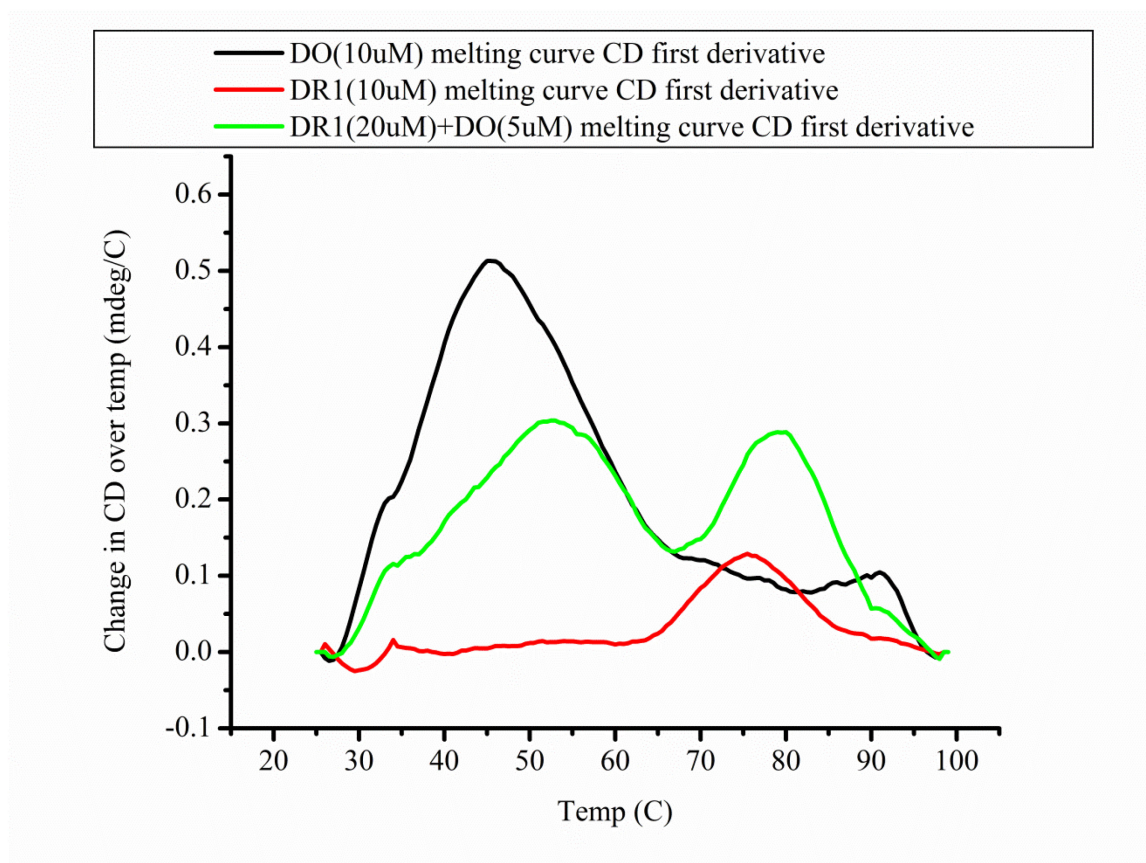
(B) Derivative of the CD scan, top of each peak represents the melting point of each sample transition.

**A**





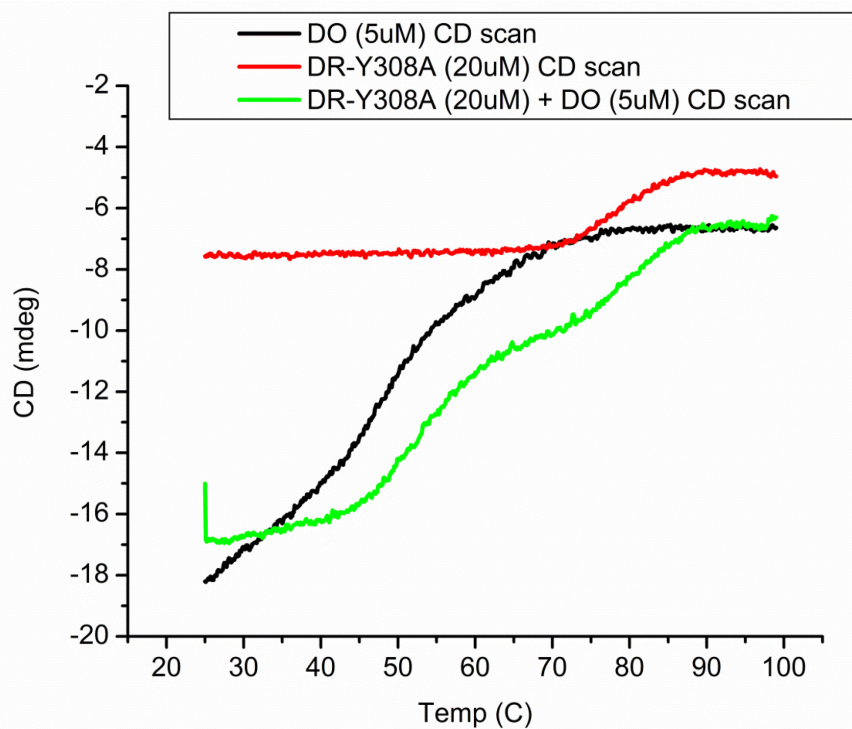
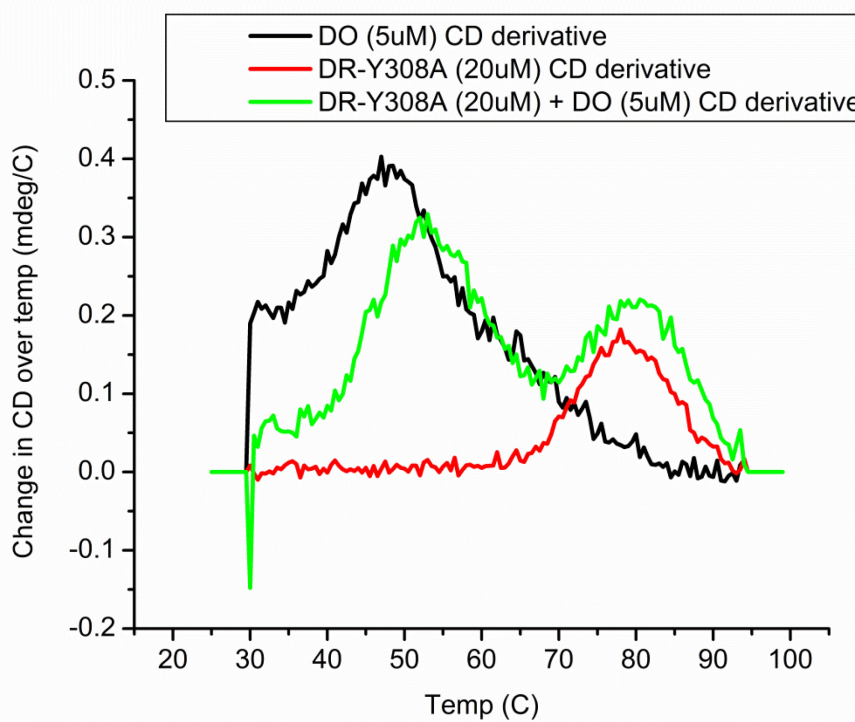
**B**



**Figure 2. Circular dichroism melting curve scan of DR1-HA(Y308A) in the presence of DO**

(A) CD scan of 20  $\mu$ M DR1 alone, 5  $\mu$ M DO alone and 20  $\mu$ M DR1 together with 5  $\mu$ M DO over a temperature range of 25  $^{\circ}$ C to 100  $^{\circ}$ C, with an increase rate of 1  $^{\circ}$ C/min.

(B) Derivative of the CD scan, top of each peak represents the melting point of each sample transition.

**A****B**

## Curriculum Vita

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## **Publications:**

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